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Cd8+ T Cell Effector Function And Transcriptional Regulation During Hiv Pathogenesis

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Abstract
A detailed understanding of the immune response to human immunodeficiency virus (HIV) infection is needed to inform prevention and therapeutic strategies that aim to contain the AIDS pandemic. The CD8+ T cell response plays a critical role in controlling viral replication during HIV infection and will likely need to be a part of any vaccine approach. The qualitative feature of the CD8+ T cell response most closely associated with immunologic control of HIV infection is its cytotoxic capacity. The pore-forming protein, perforin, is a major determinant of the cytotoxic capacity of CD8+ T cells. In the context of chronic HIV infection, enhanced perforin expression by HIV-specific CD8+ T cells is associated with greater control over HIV replication. However, individuals experiencing chronic progressive infection (CP) often demonstrate a diminished ability to express this important cytolytic molecule. HIV-specific CD8+ T cells from CP also express lower levels of the T-box transcription factor T-bet, an upstream regulator of CD8+ T cell effector differentiation and function. Whether HIV-specific CD8+ T cells from progressors possess effector capacity during the earliest stages of infection and subsequently lose it remains unclear. The relationship between perforin, T-bet, and the closely related transcription factor eomesodermin (Eomes) also remains largely undefined in the context of acute, chronic, or controlled HIV infection. In this work, we report that CD8+ T cell responses had high cytotoxic potential during acute HIV infection but perforin expression quickly waned with the resolution of peak viremia. Importantly, perforin was maintained in HIV-specific CD8+ T cells with high levels of T-bet, but not necessarily in a population of T-betLo HIV-specific CD8+ T cells that expanded as infection progressed. During chronic infection there was a generalized increase in perforin expression for both total memory and HIV-specific CD8+ T cells that was dissociated from both T-bet and Eomes. Of note, however, individuals in which perforin remained closely associated with T-bet demonstrated greater in vivo control of HIV replication. Collectively, our data imply that loss of transcriptional regulators responsible for driving strong cytotoxic responses, such as T-bet, contributes to CD8+ T cell dysfunction during chronic progressive HIV infection.

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DEDICATION

I dedicate this work to my parents, Kim and Ken, my wife, Brenda, and to my sons, Aiden and Xane, for their continuous love and support during the course of my graduate studies. I am eternally grateful for their presence in my life.

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ABSTRACT

CD8$^+$ T CELL EFFECTOR FUNCTION AND TRANSCRIPTIONAL REGULATION DURING HIV PATHOGENESIS

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A detailed understanding of the immune response to human immunodeficiency virus (HIV) infection is needed to inform prevention and therapeutic strategies that aim to contain the AIDS pandemic. The CD8$^+$ T cell response plays a critical role in controlling viral replication during HIV infection and will likely need to be a part of any vaccine approach. The qualitative feature of the CD8$^+$ T cell response most closely associated with immunologic control of HIV infection is its cytotoxic capacity. The pore-forming protein, perforin, is a major determinant of the cytotoxic capacity of CD8$^+$ T cells. In the context of chronic HIV infection, enhanced perforin expression by HIV-specific CD8$^+$ T cells is associated with greater control over HIV replication. However, individuals experiencing chronic progressive infection (CP) often demonstrate a diminished ability to express this important cytolytic molecule. HIV-specific CD8$^+$ T cells from CP also express lower levels of the T-box transcription factor T-bet, an upstream regulator of CD8$^+$ T cell effector differentiation and function. Whether HIV-specific CD8$^+$ T cells from progressors possess effector capacity during the earliest stages of infection and subsequently lose it remains unclear. The relationship between perforin, T-bet, and the closely related transcription factor eomesodermin (Eomes) also remains largely

undefined in the context of acute, chronic, or controlled HIV infection. In this work, we report that $CD8^+$ T cell responses had high cytotoxic potential during acute HIV infection but perforin expression quickly waned with the resolution of peak viremia. Importantly, perforin was maintained in HIV-specific $CD8^+$ T cells with high levels of T-bet, but not necessarily in a population of T-bet^{Lo} HIV-specific $CD8^+$ T cells that expanded as infection progressed. During chronic infection there was a generalized increase in perforin expression for both total memory and HIV-specific $CD8^+$ T cells that was dissociated from both T-bet and Eomes. Of note, however, individuals in which perforin remained closely associated with T-bet demonstrated greater *in vivo* control of HIV replication. Collectively, our data imply that loss of transcriptional regulators responsible for driving strong cytotoxic responses, such as T-bet, contributes to $CD8^+$ T cell dysfunction during chronic progressive HIV infection.

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CHAPTER 1

INTRODUCTION

Overview

A recent UNAIDS report estimates that there are currently 36.7 million people infected with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome [AIDS](UNAIDS, 2016). With almost 2 million deaths each year from AIDS-related illnesses, HIV/AIDS remains one of the leading causes of death globally. While extensive prevention education initiatives and therapeutic intervention have contributed to reducing incidents of HIV infection and mortality over the last decade, the number of new infections remains high at more than 2.1 million new infections annually (UNAIDS, 2016). These data highlight the continued need for an effective vaccine that can either prevent new infection or improve clinical outcome.

There are three potential strategies for a HIV vaccine design: a humoral approach, a cellular approach or a combination of the two. While initial attempts to induce protection via humoral responses (AIDSVAX B/B and AIDSVAX B/E) or cellular responses (STEP trial) provided little to no protection (Buchbinder et al., 2008; Gilbert et al., 2005; Pitisuttithum et al., 2006), the Thai RV144 vaccine trial, which aimed to elicit both humoral and cellular immunity, met with partial success and provided the first evidence that it may be possible to protect against HIV acquisition (Rerks-Ngarm et al., 2009). However, the RV144 vaccine strategy failed to elicit strong neutralizing antibody activity or $CD8^+$ T cell responses (Haynes et al., 2012; Montefiori et al., 2012). In addition, the protective effects were modest and of limited durability, and there is some

question as to whether the results are generalizable to groups with greater risk of HIV acquisition than the cohort examined in the RV144 trial (McMichael and Haynes, 2012). It will take several years to determine if the RV144 ALVAC-HIV/AIDSVAX B/E approach is truly protective or can be improved upon, and it is important that alternate vaccine strategies be pursued to either complement the ALVAC-HIV/AIDSVAX B/E vaccine or replace it in the case of its failure.

A truly efficacious HIV vaccine will likely need to induce several arms of the immune system, including antiviral cellular responses mediated by $CD8^+$ T cells. As such, a detailed understanding of the properties of $CD8^+$ T cells that correlate with virologic control is essential to focus vaccine development on strategies that will elicit appropriate cellular responses. The recent failure of the Merck STEP trial appeared to suggest that $CD8^+$ T cell responses incapable of preventing infection nor lower viral set-point following infection (McElrath et al., 2008). However, there is substantial correlative evidence indicating $CD8^+$ T cell responses play a significant role in controlling HIV infection at some level, if not completely (Borrow et al., 1994; Borrow et al., 1997; Carrington and O'Brien, 2003; Dalmasso et al., 2008; Goonetilleke et al., 2009b; Koup et al., 1994; Salazar-Gonzalez et al., 2009). Additionally, recent data from preclinical rhesus macaque studies suggests that $CD8^+$ T cells induced by vaccination can indeed provide some degree of protection from SIV infection (Barouch et al., 2012; Hansen et al., 2011; Hansen et al., 2013a; Hansen et al., 2009; Liu et al., 2009), supporting the idea that if strong responses in the right state of activation and anatomical location can be induced they could be effective. Together, these studies tell us we must extend our understanding of $CD8^+$ T cell responses beyond the examination of a single

function, such as IFN-γ, to define the optimal measures on which to infer vaccine efficacy. In this work we examined $CD8^+$ T cell cytotoxic potential, the transcription factors that drive this effector state, and their relationship with *in vivo* control of HIV replication in the hope of identifying useful targets for development of novel immunotherapeutics as well as vaccines.

Biology and pathogenesis of Human Immunodeficiency Virus

It is believed that the AIDS epidemic started in the mid- to late 1970s although it was not recognized as a new disease until 1981 when a growing number of young, previously healthy individuals began succumbing to an unusual opportunistic infection (*Pneumocystis carinii pneumonia*) and presenting with Kaposi Sarcoma (Hymes et al., 1981; Masur et al., 1981). In addition to the rare diseases, afflicted individuals demonstrated marked depletion of $CD4^+$ T cell from their peripheral blood. By late 1982 epidemiologic evidence indicated AIDS was an infectious disease (CDC, 1982). In 1983 researchers were able to isolate an infectious agent from the inflamed lymph nodes of a patient and found the agent possessed many characteristic of retroviruses (Barre-Sinoussi et al., 1983). Further study of the retrovirus showed that it was cytopathic to PBMC, targeting $CD4^+$ T cells in particular (Klatzmann et al., 1984a). Morphologic and genetic studies placed the retrovirus in the genus *Lentivirus* and in 1986 it was finally called human immunodeficiency virus, or HIV (Coffman 1986).

Consistent with the loss of $CD4^+$ T cells from infected individuals as well as the preferential targeting of $CD4^+$ cells in culture, the CD4 transmembrane protein was itself identified as the primary cell-surface receptor of HIV (Dalgleish et al., 1984; Klatzmann

et al., 1984b). However, it was soon realized that CD4 alone was not sufficient for entry into target cells. It took an additional twelve years before chemokine receptor type 5 (CCR5) and CXC chemokine receptor type 4 (CXCR4) were determined to function as the principal coreceptors for the virus (Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). Engagement of CD4 and chemokine receptor by the surface envelope (Env) glycyprotein on HIV allows the viral membrane to fuse with the target cell membrane resulting in the microinjection of the viral core (Ray and Doms, 2006). Once inside, the viral reverse transcriptase enzyme initiates the generation of a double-stranded DNA version of the HIV RNA genome. The newly formed DNA copy of the viral genome then enters the nucleus of the cell and is permanently integrated into the chromosomal DNA. Once integrated, HIV largely relies on host cell machinery to replicate and generate new virions.

The disease course following infection with HIV is typically broken down into three phases: (1) the acute, (2) asymptomatic, and (3) AIDS. During the acute phase, which takes place within the first few weeks following infection, there are massive levels of viral replication and a concomitant depletion of $CD4^+$ T cells both in the gastrointestinal tract and in the peripheral blood (Brenchley et al., 2004; Daar et al., 1991). The first immune responses to HIV are also detectable during this time, but typically prove to be insufficient to completely control the virus. Following the resolution of peak viral replication, infection enters the asymptomatic phase during which viral load achieves a steady state of around 10^3 to 10^4 copies of HIV RNA per ml of plasma. Although infected individuals remain outwardly healthy during this time, internally the remaining pool of $CD4^+$ T cells is gradually being depleted. After an indeterminate

amount of time, but typically within 5-10 years, the immune systems of untreated patients collapse completely thereby exposing them to attack from opportunistic infections and other diseases typically controlled in healthy individuals. It is thought that loss of the support CD4+ T cells provide to sustain immune defenses ultimately leads to this collapse and increased vulnerability (Rowland-Jones, 2003).

$CD8^+$ T cell function

CD8+ T cells are an integral part of the host immune defense against intracellular pathogens. Antigen-specific $CD8^+$ T cells are heterogeneous populations capable of performing multiple functions. Several studies have demonstrated this heterogeneity during the responses to HIV-1, CMV, and EBV infections (Appay et al., 2000; Catalina et al., 2002; Gillespie et al., 2000; Hamann et al., 1997; Makedonas et al., 2010). These responses include production of cytokines and chemokines, cytolytic effector molecules and antigen-specific lysis of major histocompatibility complex (MHC) class I matched target cells. The majority of responding $CD8^+$ T cells exert multiple functions following stimulation, but they can also respond with as little as one (depending on the number of parameters measured). Many of these functions are readily detectable by ELISpot or flow cytometry, and play specific, and potentially differential, roles in immunity against a variety of viruses. Several of the functions associated with $CD8^+$ T cells that are frequently assessed in response to HIV infection are described briefly below:

Interferon (IFN)-γ

IFN-γ is the only member of the type II class of interferons, a family of cytokines originally discovered for their ability to interfere with influenza virus replication (Isaacs and Lindenmann, 1957). IFN-γ is the single most commonly used function to assess $CD8^+$ T cell responses to infection or vaccination. It promotes a general antiviral state by inducing the conversion of the constitutive proteasome to the immunoproteasome (Groettrup et al., 1996), upregulating expression of the TAP transporter proteins (Cramer et al., 2000; Epperson et al., 1992), and increasing expression and stability of MHC class I molecules (Johnson and Pober, 1990; Wallach et al., 1982). In some contexts, IFN-γ also increases the susceptibility of virally infected cells to apoptosis by increasing the expression of the TNF-α receptors and Fas/FasL (Tsujimoto et al., 1986; Xu et al., 1998). However, IFN-γ may also enhance HIV replication (Biswas et al., 1992). Thus in the context of HIV infection IFN-γ is potentially both beneficial and detrimental to inhibiting viral replication.

Interleukin (IL)-2

IL-2 is the primary growth factor for T cells (Dinarello and Mier, 1986). Although typically considered a $CD4^+$ T cell cytokine, $CD8^+$ cells are also quite capable of producing IL-2 (Zimmerli et al., 2005). It has no direct antiviral effector function, but it does promote expansion of $CD4^+$ and $CD8^+$ cells, thereby amplifying the effector response to pathogens (Seder et al., 2008). IL-2 may also be important for programming $CD8^+$ T cells for better memory generation and effector function (Williams et al., 2006). IL-2 production by $CD8^+$ T cells is correlated with proliferation of $CD8^+$ cells

independent of $CD4^+$ T cell IL-2 production (Zimmerli et al., 2005), and both IL-2 production and proliferation are preserved in nonprogressive HIV infection (Betts et al., 2006; Migueles et al., 2002). However, IL-2-induced activation and proliferation of $CD4^+$ T cells may also increase the availability of target cells for infection as well as increase viral replication by infected cells (Davey et al., 1997).

Tumor necrosis factor (TNF)-α

TNF-α is a member of the TNF superfamily and was first identified by its ability to induce necrosis in solid tumors (Carswell et al., 1975). It has subsequently been shown to be an important antiviral factor due to its role as a mediator of apoptosis as well as inflammation and immunity (Aggarwal, 2003; Kull, 1988; Lazdins et al., 1997). TNF-α is initially expressed as a biologically active homotrimer on the cell surface that the matrix metalloprotease TNF-α converting enzyme can subsequently cleave into its soluble form (Black et al., 1997). Soluble TNF-α preferentially binds TNF-RI, which, upon being bound, initiates a signaling cascade that induces apoptosis of infected cells. Membrane bound TNF-α binds TNF-RII and plays an important role in driving NF-κB activation and inflammation (Chen and Goeddel, 2002; Wajant et al., 2003b). TNF-α also promotes an antiviral state by enhancing expression of MHC class I and by inducing expression of IL-12 and IL-18 which are both important for upregulating production of IFN-γ by $CD8^+$ T cells (Feldmann et al., 1995; Johnson and Pober, 1990; Scheurich et al., 1986). However, similar to IFN-γ and IL-2, activation of cells induced by TNF-α can also result in increased production of virus (Duh et al., 1989; Folks et al., 1989; Harrer et al., 1993).

Chemokines

Both $CD4^+$ and $CD8^+$ T cells secrete a variety of chemotactic cytokines (chemokines) upon activation (Rollins, 1997). Chief among them are the β-chemokines macrophage inflammatory protein-1α (MIP-1α) and -1β (MIP-1β) and regulated upon activation normal T cell expressed and secreted (RANTES). MIP-1α and MIP-1β can be found in cytotoxic granules while RANTES is stored in a separate secretory compartment called the RANTES secretory vesicle (RSV) (Catalfamo et al., 2004; Wagner et al., 1998). Both types of granules are rapidly released following T cell activation. New MIP-1α and MIP-1β synthesis occurs within a few hours of activation, while RANTES can take several days to be upregulated following its initial release. All three contribute to an inflammatory response primarily by recruiting leukocytes to the site of injury or infection.

β-chemokines were the first noncytotoxic factors secreted by $CD8^+$ T cells to be identified that directly inhibit HIV replication (Cocchi et al., 1995). They inhibit replication *in vitro* by binding their cognate chemokine receptor, CCR5, which serves as a coreceptor for viral binding and entry into target cells. Binding of β-chemokines to CCR5 is thought to block access to and induce the internalization of the receptor (Copeland, 2002). The exact role the β-chemokines play during HIV infection *in vivo* may still be a matter for debate. β-chemokines do not appear to prevent infection of monocytes and may actually enhance viral replication in these cells (Dragic et al., 1996; Moriuchi et al., 1996; Schmidtmayerova et al., 1996). RANTES (but not MIP-1α or MIP-1β) can increase attachment of HIV to cells in a manner independent of both CD4 and CCR5 and increase replication by activating signal transduction pathways (Trkola et al.,

1999). Serum β-chemokine concentrations do not correlate with HIV disease status, although patients with progressive infection tend to have higher levels than those with non-progressive infection (Saha et al., 1998). There is also the suggestion that physiologic levels of β-chemokines are not high enough to exert anti-HIV activity (Mackewicz et al., 1997), although there is the possibility that concentrations are sufficient for inhibition in the microenvironment of the $CD8^+$ T cell. Thus, while these molecules have been shown to have inhibitory effects *in vitro*, they may in fact fuel infection *in vivo* by not only recruiting uninfected target cells to sites of active viral replication but also by enhancing infection of those cells.

$CD8^+$ T cell antiviral factor (CAF)

CAF was originally defined in the context of HIV infection and the demonstration of its activity provided the first indication that $CD8^+$ T cells possess the ability to inhibit HIV replication (Walker et al., 1986). CAF is a noncytolytic, diffusible lymphokine that lacks identity with IFN-α, IFN-β, TNF-α, IL-4, IL-6 or the β-chemokines MIP-1α, MIP-1β and RANTES (Leith et al., 1997; Mackewicz et al., 1994; Rubbert et al., 1997; Walker and Levy, 1989). Aside from, there is little known and much debate about the exact nature of CAF (Chang et al., 2003; Mackewicz et al., 1994; Vella and Daniels, 2003). It may be the activity of one or more cytokines or chemokines acting together, or it could be an as yet unidentified molecule (Chang et al., 2002). In the case of HIV, CAF appears to function by suppressing HIV long terminal repeat (LTR)-mediated gene expression in $CD4^+$ T cells (Copeland et al., 1997). It does not block HIV entry (Copeland et al., 1997), proviral integration (Mackewicz et al., 2000), or reverse transcription (Chang et al., 2003), nor is

it MHC class I restricted (Vella and Daniels, 2003). Due to CAF activity being neither HIV-antigen specific nor produced only by $CD8^+$ T cells has led to the hypothesis that it may in fact be part of an innate rather than an adaptive immune response (Chang et al., 2002; Le Borgne et al., 2000). Despite this, the suppressive capacity of CAF appears to be real and further investigation is warranted to determine its identity.

Cytotoxicity

Perhaps the most important function of $CD8^+$ T cells is to recognize and kill infected cells. This function has been shown to be important for control of several infections, including EBV (Callan, 2003), CMV (Belz and Doherty, 2001; Gillespie et al., 2000), HBV (Guidotti et al., 1996), and HCV (Lechner et al., 2000). $CD8^+$ T cells predominantly mediate killing through the secretion of granzymes and perforin (Peters et al., 1991; Shankar et al., 1999). Granzymes are serine proteases that cleave caspases to induce apoptosis (Bots and Medema, 2006; Heusel et al., 1994) and perforin is a pore-forming protein that is required for delivery of granzymes into a target cell (Bolitho et al., 2007; Voskoboinik et al., 2006). Both of these proteins are contained within lytic granules and are released early after $CD8^+$ T cell activation into the immunological synapse formed between the $CD8^+$ T cell and a target cell. This process of degranulation is MHC class I restricted and antigen specific and likely plays an important role in control of viral infection *in vivo* (Trambas and Griffiths, 2003).

$CD8^+$ T cells can also mediate killing by the Fas-Fas ligand (FasL) pathway. FasL is upregulated by $CD8^+$ T cells following activation by a target cell (Rouvier et al., 1993). Cross-linking of membrane bound FasL and the cell surface death receptor Fas expressed

on targets cells induces assembly of an intracellular death-inducing signaling complex (DISC) (Kischkel et al., 1995). DISC formation causes activation of a caspase cascade that ultimately leads to apoptosis of the target cell. Individual CTL are thought to be capable of both FasL- and perforin-mediated killing (He and Ostergaard, 2007), however, cytolysis of HIV-infected target cells appears to be largely perforin-mediated with no clear evidence of a contribution of FasL-mediated killing by HIV-specific $CD8^+$ T cells (Shankar et al., 1999). In addition, reports that a soluble form of FasL can not only block apoptosis but also induce proliferation and NF-κB activation of HIV target cells raises the possibility that its role in infection is not always directly antagonistic (LA et al., 2009; Wajant et al., 2003a).

Assessing $CD8^+$ T cell antiviral responses

Early studies of $CD8^+$ T cell response to HIV employed cytotoxicity assays, such as the chromium release assay (CRA), to measure HIV-specific CTL activity. The CRA was used to show that HIV-specific $CD8^+$ T cells have direct cytotoxic effects against HIV-infected $CD4^+$ T cells (Walker et al., 1987) and was important in establishing the link between emergence of HIV-specific CTL and resolution of peak viremia during primary HIV infection (Koup et al., 1994). However, questions were raised about the ability of the assay to link CTL activity and HIV viral load during chronic infection (Ogg et al., 1998). The CRA is also laborious, relatively insensitive, highly variable, and, most importantly, provides little information about the cytolytic $CD8^+$ T cells themselves other than that they can kill.

Direct detection and quantification of antigen-specific $CD8^+$ T cells by MHC class I tetramer technology or IFN-γ production offered more rapid, more sensitive and less variable assays than measurement of cytotoxicity by the CRA. While an inverse relationship between simple frequency of HIV-specific $CD8^+$ T cells and plasma viral load was initially established on the basis of tetramer staining (Ogg et al., 1998), this finding was not supported by subsequent studies that found no relationship between the frequency of IFN-γ producing HIV-specific $CD8^+$ T cells and HIV viral load (Addo et al., 2003; Betts et al., 2001; Gea-Banacloche et al., 2000). It was proposed that these disparate findings were the result of a significant portion of the circulating tetramer-staining $CD8^+$ T cell population being functionally impaired (Kalams and Walker, 1998; Zajac et al., 1998). While early studies in this area have shown that the majority of tetramer-positive HIV-specific $CD8^+$ T cells can produce IFN-γ (Appay et al., 2000; Goulder et al., 2000), high expression of inhibitory markers including PD-1, CD160, 2B4, and Lag-3 on HIV-specific CD8+ T cells may indicate some degree of functional insufficiency (Blackburn et al., 2009; Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Yamamoto et al., 2011).

IFN-γ was presumed to be an antiviral marker largely because $CD8^+$ T cell clones that produced it early after stimulation became CTLs after further culture (Jassoy et al., 1993; Morris et al., 1982). This, combined with its ease of measurement by ELISpot or flow cytometry, made IFN-γ a popular choice for detecting HIV-specific cellular responses in both HIV-infected individuals and participants in HIV vaccine trials. However, studies have demonstrated the inadequacy of IFN-γ as a surrogate marker for HIV control. One study found a positive correlation between frequency of IFN-γ-

producing HIV-specific $CD8^+$ T cells and HIV viral load (Betts et al., 2001), and the failure of the STEP trial further highlighted the misinterpretation of IFN-γ as a surrogate of protection: 77% of vaccinees had an IFN-γ response to one or more HIV antigens by ELISpot, yet there was no protection from infection or enhanced viral control following infection (McElrath et al., 2008). While increased rates of escape mutations within vaccine-targeted $CD8^+$ T cell epitopes indicate that vaccine-induced immune pressure was exerted on the virus, the nature of the selective forces has not been defined (Rolland et al., 2011). More recently, studies have shown more directly that the magnitude of IFN-γ responses does not correlate with $CD8^+$ T cell HIV inhibitory activity (Chen et al., 2009; Yang et al., 2012). Thus, while IFN-γ may be a good indicator of the presence of a response, it cannot be used alone to infer the anti-HIV capacity of T cells.

As discussed earlier, responding $CD8^+$ T cell have the capacity to produce several different functions, and measuring a single function such as IFN-γ likely does not describe the true extent of an antigen-specific immune response. This idea was confirmed by studies based on the murine LCMV model of infection. The Armstrong strain of LCMV is cleared following acute infection while the clone 13 strain results in chronic infection. Following resolution of acute infection by the Armstrong strain, a subset of LCMV-specific IFN-γ-producing $CD8^+$ T cells emerges that also produces IL-2 (Kristensen et al., 2002). This same subset does not appear following chronic infection with LCMV clone 13, as LCMV-specific cells continue to produce only IFN-γ. Wherry *et al.* extended these findings to show that $CD8^+$ T cells are not only multifunctional, but that functional capacity of LCMV-specific $CD8^+$ T cells is gradually lost in the context of chronic clone 13 infection (Wherry et al., 2003). Cells producing IL-2 were the first

functional subset lost, followed by those producing TNFα, while IFN-γ was the most resistant to this "functional exhaustion". These studies demonstrate that individual $CD8^+$ T cells are capable of responding with multiple functions simultaneously and that measuring IFN-γ alone fails to exclude T cells that are potentially impaired in their functional capacity. They also indicate that including more than one functional marker during the assessment of cellular responses to infection or vaccination will identify cells with greater antiviral potential.

Appay *et al.* and Sandberg *et al.* were the first to demonstrate the functional complexity of human $CD8^+$ T cell responses (Appay et al., 2000; Sandberg et al., 2001). They examined cellular responses to either HIV and CMV (Appay et al., 2000) or CMV alone (Sandberg et al., 2001) and showed that different cells specific for the same antigen were capable of producing TNF-α, IFN-γ and MIP-1β or IL-2, TNF-α and IFN-γ, respectively. Technical limitations prevented either of these studies from examining the capacity of individual antigen-specific cells to co-produce cytokines or chemokines, however, advances in flow cytometry technology allowing the concurrent measure of up to 18 functional and phenotypic markers soon provided a new tool for assessment of responses (Perfetto et al., 2004). The first true demonstration of multifunctional T cells in humans came when De Rosa *et al.* examined responses of antigen-specific T cells elicited by HBV- and HIV-vaccination or natural infection, measuring five different functions (IL-2, TNF-α, IFN-γ, MIP-1β and IL-4) simultaneously (De Rosa et al., 2004). Both $CD4^+$ and $CD8^+$ T cells displayed a surprising breadth and complexity of responses that could not have been captured by the measurement of any single function alone. Antigen-specific $CD8^+$ T cells were capable of producing IL-2, TNF-α, IFN-γ, and MIP-1β alone

or in combination. Importantly, there were several functional subsets that did not include IFN-γ production and thus would have been missed had IFN-γ been measured alone. This study established that measurement of multiple functions provides a more sensitive and complete evaluation of T cell responses elicited by vaccination or natural infection. It also presented the possibility that distinct functional expression patterns might provide correlates of protection or disease progression.

Immune correlates of control of HIV

Untreated HIV infection typically results in a chronic progressive disease that culminates in AIDS within 10 years, but a subset of less than 1% of infected individuals spontaneously control viral replication to undetectable levels by standard clinical assays in the absence of therapy. This group of so-called "elite controllers" (ECs) experience very slow rates of $CD4^+$ T cell decline and rarely progress to AIDS (Sedaghat, 2009; Migueles and Connors, 2010; Sajadi, 2009). Because of this ECs have been studied extensively to better understand the mechanism(s) responsible for their enhanced capacity to suppress HIV replication. It was hoped that by comparing the immune responses of ECs to those of individuals with chronic progressive infection (CPs) targets could be identified that would facilitate the design of vaccines or therapeutic strategies to prevent or control infection.

Innate immunity

There is evidence that myeloid dendritic cells (DCs) from ECs have enhanced antigen-presenting capabilities (Huang et al., 2010). They also demonstrate lower levels of pro-

inflammatory cytokine secretion. These characteristics make them potent inducers of $CD8^+$ T cell responses without also contributing to a state of generalized immune activation. Plasmacytoid DCs are the main source of the cytokine IFN-α, an important modulator of innate restriction factors and a potent enhancer of $CD8^+$ T cell and natural killer (NK) cell responses (Bosinger and Utay, 2015; Urban et al., 2016). Plasmacytoid DCs are maintained at levels similar to HIV-negative individuals whereas they are depleted in the peripheral blood of individuals with progressive infection (Barblu et al., 2012; Machmach et al., 2012). Despite these differences between ECs and CPs, there is no clear evidence that either DC subset makes a significant contribution to control or to HIV pathology.

NK cells target and kill infected cells non-specifically via killer immunoglobulin receptors (KIRs). Expression of certain KIRs, such as KIR3DS1 and its ligand BW4, is associated with slower progression of HIV disease (Martin et al., 2007). However, these same KIRs were not found to be overrepresented in a group of ECs making their contribution to control ambiguous (O'Connell et al., 2009). In addition, while there is evidence of viral sequence evolution as a result of NK-mediated pressure (Alter et al., 2011), the *in vitro* antiviral activity of NK cells from ECs was found to be relatively weak (O'Connell et al., 2009). Finally, experimental depletion of NK cells during either acute or chronic infection in the nonhuman primate SIV model had no impact on the kinetics of viral replication(Choi et al., 2008a; Choi et al., 2008b). It should be noted, however, that an antibody against CD16 was used to deplete NK cells in these experiments and not all NK cells express CD16 in nonhuman primates. Together these

studies indicate innate immunity plays an important role in overall immunity to HIV infection but there is little evidence to correlate NK cells or DC responses with control.

Humoral immunity

B cells produce antibodies to fight invading pathogens. Antibodies mediate their protective effects in many ways, including through antibody-dependent cell-mediated cytoxicity (ADCC) or through neutralization, a process in which the antibody sterically hinders the pathogen-host interaction. One recent report indicated ECs might have increased ADCC capacity (Lambotte et al., 2009), but this could not be confirmed in a follow-up study (Smalls-Mantey et al., 2012). Neutralizing antibodies can arise early infection, select for Env escape variants, and impede viral replication (Bar et al., 2012; Liao et al., 2013; Mahalanabis et al., 2009; Wei et al., 2003). However, increased range of neutralizing antibody specificities is typically associated with high viral loads and as such they are rarely found in EC (Bailey et al., 2006; Deeks et al., 2006; Doria-Rose, 2010; Lambotte et al., 2009; Mahalanabis et al., 2009; Pereyra et al., 2008; Sather et al., 2009). In addition, the development of highly potent broadly neutralizing antibodies capable of targeting multiple HIV variants can take two to four years and likely require the continued presence of moderate to high levels of viremia (Burton and Mascola, 2015). Together these studies suggest antibody responses play a limited role in the control of viral replication in ECs.

CD4+ T cells

$CD4^+$ T cells are a critical component of adaptive immune responses to infection given the support they provide to developing B cells and $CD8^+$ T cells. However, $CD4^+$ T cells are also the primary targets of HIV and their progressive loss over the course of infection is linked to development of opportunistic infections and CD8+ T cell dysfunction (Altfeld and Rosenberg, 2000; Douek et al., 2002; Kalams et al., 1999). CD4+ T cells from EC are more functional, with greater frequency of cells able to produce IL-21 or simultaneously express IL-2 and IFN-γ (Chevalier et al., 2011; Emu et al., 2005; Lichterfeld et al., 2004; Pereyra et al., 2008). CD4+ T cell responses are largely similar when comparing ECs and antiretroviral therapy suppressed individuals, however, leading some to question whether differences between EC and CP $CD4^+$ T cells are a consequence or cause of controlled viral replication (Tilton et al., 2007). One recent study found a correlation between acute phase $CD4^+$ T cell cytotoxic capacity and set point viral loads, but it remains to be determined if a similar phenomenon occurs in individuals with EC status (Soghoian et al., 2012). It is also unclear if targeting of specific $CD4^+$ T cell epitopes is associated with control. Thus, although there is little current evidence to suggest it, a direct role for $CD4^+$ T cells in suppressing HIV replication cannot be ruled out entirely.

CD8+ T cells

There is strong correlative and direct evidence that $CD8^+$ T cells are critical for control of HIV replication. This comes from both HIV infection in humans and simian immunodeficiency virus (SIV) infection in nonhuman primates (NHP). First, the

resolution of peak viremia during acute HIV infection is temporally associated with the expansion of HIV-specific $CD8^+$ T cells (Borrow et al., 1994; Koup et al., 1994). Second, immunologic pressure exerted by HIV- and SIV-specific $CD8^+$ T cells is linked to the emergence of viral escape mutations during acute and chronic infection (Allen et al., 2000; Borrow et al., 1997; Evans et al., 1999; Goonetilleke et al., 2009b; Salazar-Gonzalez et al., 2009). Third, there is a strong correlation between specific MHC class I alleles and non-progressive infection in both humans and rhesus macaques (Carrington and O'Brien, 2003; Dalmasso et al., 2008; Goulder et al., 1997; Loffredo et al., 2007; Yant et al., 2006). Finally, experimental depletion of $CD8^+$ T cells in SIV-infected rhesus macaques results in a concomitant loss of control of viral replication (Jin et al., 1999; Schmitz et al., 1999).

Historically, neither the quantity nor the breadth of the HIV-specific $CD8^+$ T cell response has correlated with protection. However this is based almost entirely on analysis of IFN-γ responses alone, which is likely not representative of the true response to infection. Given the capacity of $CD8^+$ T cells to produce multiple functions, it was possible that the key to protection was a matter of quality of the response rather than simply quantity. Zimmerli *et al.* were the first to show that higher quality HIV-specific $CD8^+$ T cell responses consisting of simultaneous production of IL-2 and IFN-γ, compared to IFN-γ alone, differentiate nonprogressive and progressive HIV infection, respectively (Zimmerli et al., 2005). The ability of nonprogressor $CD8^+$ T cells to produce IL-2 was consistent with the maintenance of greater proliferative capacity than for cells from progressors (Migueles et al., 2002; Zimmerli et al., 2005). This data suggested that quality of the response was indeed important. To investigate this

association further, our lab measured five T cell functions (IL-2, IFN-γ, TNF-α, MIP-1β and CD107a) simultaneously to characterize HIV-specific $CD4^+$ and $CD8^+$ T cell responses in a cohort of progressors and nonprogressors (Betts et al., 2006). This study demonstrated that while the absolute frequency of HIV-specific $CD8^+$ T cells does not correlate with viral control, the frequency of specific functional subsets does correlate with control. Comparing the functional profiles of the HIV-specific $CD8^+$ T cell responses between progressors and nonprogressors, the two groups were differentiated by a higher degree of functionality in the nonprogressors than in the progressors. A population of $CD8^+$ T cells capable of producing all five functions was observed almost exclusively in the nonprogressors, and a subset of $CD8^+$ T cells producing IFN-γ, TNF-α, MIP-1β and CD107a was also more prevalent in this group than in progressors. These responses were consistent across multiple HIV proteins (Gag, Pol, Env and Tat/Rev/Vif/Vpr/Vpu). Furthermore, within the progressor group the magnitude and proportion of the HIV-specific $CD8^+$ T cell responses positive for these same five- and four-function subsets inversely correlated with viral load. This demonstrates that by measuring multiple functions simultaneously it is possible to discern a difference between progressive and nonprogressive HIV infection based on the functional capacity of HIV-specific $CD8^+$ T cell responses.

In support of an association between polyfunctional cells and protection during infection, Darrah *et al*. demonstrated that vaccine-elicited polyfunctional $CD4^+$ T cells are far better at providing protection to mice challenged with *Leishmania major* than dualfunctional or monofunctional cells (Darrah et al., 2010; Darrah et al., 2007). However, a similar phenomenon for $CD8^+$ T cells has not been established in this or other

models of infection and the role of polyfunctional $CD8^+$ T cells in the context of HIV infection is surrounded by questions. First, how can polyfunctionality be a significant correlate of protection when only a small proportion of nonprogressor HIV-specific $CD8^+$ T cells actually produce five functions and some nonprogressors do not have this or any other measurable functional subset (Betts et al., 2006; Emu et al., 2008)? Second, why do polyfunctional HIV-specific $CD8^+$ T cells induced in progressors during antiviral therapy generally fail to impart virologic control upon cessation of therapy (Davey et al., 1999; Ortiz et al., 2001; Rehr et al., 2008)? Finally, how do IL-2, TNF-α, IFN-γ, and MIP-1β become protective in combination when they are not individually associated with protection and they all have potentially pleiotropic effects in the context of HIV infection (as discussed earlier)? Is it a synergistic effect of multiple functions acting in concert, or the ability of polyfunctional antigen-specific $CD8^+$ T cells to produce more cytokine on a per cell basis (Ferre et al., 2009; Precopio et al., 2007)? Is it simply that these cells are functional in a way not yet measured? Ultimately, it is a question of whether polyfunctional HIV-specific $CD8^+$ T cell responses are a cause or consequence of control. It is possible polyfunctionality, as defined by the above measures, is not indicative of the direct protective capacity of a cell but rather is a surrogate of a protective response. This is highlighted by the fact that vaccine studies in which polyfunctional CD8+ T cell response were induced but were unable appreciably control HIV replication.

Cytotoxicity is one function of $CD8^+$ T cells that is unequivocally antiviral, as direct killing of virally infected cells will certainly impact viral replication. Cytotoxic capacity can be assessed directly or indirectly by several different methods, each with its

own advantages and disadvantages. As discussed earlier, direct cytotoxic capacity of CTLs can be assessed *in vitro* using the chromium release assay, or by a similar fluorescence-based assay, and these assays were important for establishing the role of CTL in controlling HIV infection (Borrow et al., 1994; Koup et al., 1994; Walker et al., 1987). Although several studies have since used the CRA to show HIV-specific $CD8^+$ T cells from ECs maintain greater cytotoxicity than those from CPs (Andersson et al., 1999; Appay et al., 2000; Zhang et al., 2003), the connection between the results of these assays and the *in vivo* state has been questioned (Ogg et al., 1998). Critics of this interpretation cite the prolonged culture and expansion of cells *in vitro* required to generate sufficient numbers of CTLs for quantitative CRA (Doherty, 1998), which likely introduces bias in CTL analysis as it only measures the subset of memory and effector cells capable of proliferating and remaining functional under artificial conditions. Additionally, these assays only measure the effects of CTL on the target cells, without providing any phenotypic or functional characteristics about the CTL itself. This represents a critical loss of information when trying to determine the exact nature of these cells.

$CD8^+$ T cell cytotoxic potential can also be assessed directly by flow cytometry. Unlike the cytotoxicity assays, this method does not measure killing but rather determines killing potential based on the perforin and granzyme content of the $CD8^+$ T cell, with the concept that CD8+ T cells that do not express these markers would be incapable of killing targets. The advantage to this method is that flow cytometry permits measurement of additional parameters, thus providing a more complete picture of the cells with killing potential. Perforin content of antigen-specific $CD8^+$ T cells can be measured by staining resting cells with MHC class I tetramers or by peptide-stimulation.

When Appay *et al*. compared perforin content of CP antigen-specific $CD8^+$ T cells identified directly *ex vivo* by tetramer staining, they found that HIV-specific $CD8^+$ T cells were deficient for perforin compared to CMV-specific $CD8^+$ T cells (Appay et al., 2000). However, MHC class I tetramer staining may provide sufficient stimulation to induce perforin release, resulting in an underestimation of perforin content. Also, by measuring perforin content of resting cells, only immediate killing potential is measured and the sustainability of the cytotoxic response cannot be determined. To assess serial killing potential, CTLs had to be stimulated and perforin content measured after the cells were allowed to progress through multiple rounds of proliferation. Several studies measuring cytotoxic potential in this way suggested that HIV-specific $CD8^+$ T cells from ECs maintain the ability to upregulate perforin, a property that was deficient in CPs (Andersson et al., 1999; Appay et al., 2000; Migueles et al., 2002). While these results were in line with the results from the cytotoxicity assays, they also shared the same potential for memory subset bias introduced by the need for prolonged culture.

As a result of the difficulty in measuring cytotoxic factors in activated cells directly *ex vivo*, we developed an assay that utilized CD107a expression on $CD8^+$ T cells as a surrogate marker of killing capacity (Betts et al., 2003). CD107a lines the membranes of cytotoxic granules and is exposed on the cell surface following activation and degranulation of $CD8^+$ T cells. This technique was used recently to suggest cytotoxicity is one of the primary mechanisms responsible for both the enhanced HIV inhibitory potential of $CD8^+$ T cells from ECs as well as for the emergence of HIV escape mutants during acute infection (Ferrari et al., 2011; Freel et al., 2010; Freel et al., 2012). It is important to note, however, that although expression of CD107a correlates well with

the exocytosis of perforin and cytotoxicity (Betts et al., 2003; Hersperger et al., 2008), it does not directly equate to actual killing as not all granules contain perforin or granzymes (Wolint et al., 2004).

Through further testing, we soon discovered that the most commonly used perforin antibody (clone δG9) was sensitive to the degranulation assay, which required the use of monensin to neutralize intracellular pH (Hersperger et al., 2008). By using a different monensin-insensitive perforin antibody (clone D48) we found that $CD8^+$ T cells are capable of rapidly (within 4-6 hours) upregulating perforin following antigen-specific stimulation without the requirement for proliferation (Hersperger et al., 2008; Makedonas et al., 2009). We also found that newly synthesized perforin can traffic directly to the immunological synapse in a process that largely bypasses cytotoxic granules (Makedonas et al., 2009). This new perforin antibody thus allowed a direct assessment of antigen-specific $CD8^+$ T cell cytotoxic potential directly *ex vivo*. Used in conjunction with standard intracellular cytokine staining (ICS) we could now assess cytokine production and cytotoxic potential simultaneously.

In an attempt to better understand the HIV-specific $CD8^+$ T cell mechanisms of viral control we re-evaluated HIV-specific $CD8^+$ T cell polyfunctional responses, including the ability to rapidly upregulate perforin. Comparing the functional profiles of HIV-specific $CD8^+$ T cell responses from a cohort of elite controllers and chronic progressors we confirmed that elite controllers have a higher frequency of polyfunctional cells compared to chronic progressors based on the expression of IL-2, IFN-γ, TNF-α, MIP-1β and CD107a (Hersperger et al., 2010). HIV-specific $CD8^+$ T cells from elite controllers also have a greater ability to rapidly upregulate perforin directly *ex vivo*

compared to progressors. This identified a function of the elite controller HIV-specific $CD8^+$ T cell response that confered an enhanced ability to directly eliminate HIV-infected cells. Interestingly, however, there was no link between perforin upregulation and polyfunctionality. Rather, perforin expression was associated with cells of limited functional capacity, most frequently seen in combination with production of MIP-1α or degranulation (CD107a). Thus, while highly polyfunctional cells are a correlate of control, so too are oligofunctional cells, depending on the functions produced. The question then became which of these populations, if either, is involved in control of viremia. Freel *et al.* recently demonstrated that HIV-specific $CD8^+$ T cell capacity to inhibit HIV replication *in vitro* is correlated with the expression of MIP-1β and CD107a and not linked to upregulation of IL-2, TNF-α or IFN-γ (Freel et al., 2010). While noncytolytic mechanisms likely play some role in inhibition (Freel et al., 2012; Killian et al., 2011), given our findings it is not unreasonable to speculate that many of the cells expressing MIP-1β and CD107a are also upregulating perforin and inhibiting mainly through cytotoxicity, although this hypothesis has not been confirmed. Still, these studies only establish that HIV-specific $CD8^+$ T cells found during controlled HIV infection have greater functional capacity and inhibitory potential, they do not tell us if these responses are actively involved in virus control or they are a result of control by some other mechanism.

Transcriptional regulation of $CD8^+$ T cell differentiation and function

Understanding the underlying mechanisms involved in $CD8^+$ T cell differentiation and function may provide insight into the factors responsible for the dysfunction

observed during chronic infection. To this end, several transcription factors have been identified that regulate the transition of $CD8^+$ T cells into effector cells, including the T-box transcription factors T-box expressed in T cells (T-bet) and eomesodermin (Eomes). While the majority of our current knowledge about the transcriptional control of $CD8^+$ T cell function comes from mouse models, we and others have recently begun assessing the role these transcription factors play in human T cell effector function. Below, we discuss what we know from mouse models as well as our recent findings regarding $CD8^+$ T cell dysfunction in the context of HIV infection.

T-bet and Eomes are T-box binding transcription factors that play important roles in promoting $CD8^+$ T cell effector differentiation and function. The T-box is a 180-190 amino acid sequence highly conserved across T-box family members that acts as a DNA-binding domain. T-bet and Eomes share 74% sequence identity in their T-box domains, but lack sequence similarity outside this region. Like other T-box binding transcription factors, they mediate developmental transitions through epigenetic modifications (Miller and Weinmann, 2009).

T-bet was originally identified as a determinant of T_H1 cell lineage commitment (Szabo et al., 2000), but subsequent work demonstrated its importance also as a regulator of $CD8^+$ T cell effector differentiation and function (Joshi et al., 2007; Sullivan et al., 2003; Takemoto et al., 2006). T-bet positively regulates several genes associated with effector function, including perforin, granzyme B, β-chemokines and IFN-γ (Jenner et al., 2009), and negatively regulates genes such as IL-2 and PD-1 (Jenner et al., 2009; Kao et al., 2011; Szabo et al., 2000). Although T-bet activity was originally described as being required for $CD4^+$ and $CD8^+$ T cell IFN-γ expression and cytotoxicity, these functions

were not completely ablated in T-bet knock-out mice, suggesting the existence of a T-bet-independent regulatory mechanism (Sullivan et al., 2003; Szabo et al., 2002). This observation led to the discovery that $CD8^+$ T cells also express Eomes, which shares both redundant and reciprocal functions with T-bet (Pearce et al., 2003). The genes under the control of Eomes have not been as well defined as for T-bet, but knockdown of Eomes in activated $CD8^+$ T cells causes decreased expression of IFN-γ, perforin, and granzyme B, indicating there is at least some overlap with T-bet in promoting effector function. Combined T-bet and Eomes deficiency results in the loss of CTL identity and anomalous production of IL-17 by $CD8^+$ T cells (Intlekofer et al., 2008), suggesting that T-bet and Eomes coordinately regulate CTL differentiation.

Conversely, T-bet and Eomes appear to play opposing roles in the generation of memory $CD8^+$ T cells. Both transcription factors cause the upregulation of IL-2Rβ (CD122), which is required for long-term memory $CD8^+$ T cell survival and homeostatic proliferation in response to IL-15 signals (Intlekofer et al., 2005). However, whereas high expression of Eomes correlates with a central memory (T_{CM}) phenotype, high expression of T-bet represses IL-7Rα expression to drive formation of effector (T_E) and effector memory (T_{EM}) subsets at the expense of T_{CM} cell generation (Banerjee et al., 2010; Rao et al., 2010). T-bet is highest in early effector $CD8^+$ T cells but progressively declines as memory cells form while Eomes is initially upregulated in early effector $CD8^+$ T cells, but increases during the effector to memory transition (Joshi et al., 2011). These divergent expression patterns may be partly explained by the way these transcription factors are induced. T-bet is rapidly induced in activated $CD8^+$ T cells downstream of TCR signaling and augmented by inflammatory signals such as IL-12 (Joshi et al., 2007;

Takemoto et al., 2006). Expression of T-bet initiates a positive feedback loop by upregulating IL-12Rβ, thereby increasing sensitivity to additional IL-12. Eomes induction is less well defined. However, studies indicate it is induced subsequent to T-bet, amplified by IL-2 and repressed by IL-12 (Takemoto et al., 2006). This suggests the differential expression patterns of T-bet and Eomes in $CD8^+$ T cells, and in turn the differentiation state, depends on the degree of inflammation in the immediate environment of the cell.

Transcription factors in murine models of chronic infection

As discussed earlier, normal differentiation and function of virus-specific $CD8^+$ T cells is altered during chronic infection and exposure to persistent antigen. Effector cells fail to form functional memory populations and instead gradually become more exhausted. A few transcription factors have been shown to play a role in this process, including Blimp-1, T-bet and Eomes (Kao et al., 2011; Paley et al., 2012; Shin et al., 2009; Wherry et al., 2007). Blimp-1 drives effector differentiation during acute infection; however, in the context of chronic infection Blimp-1 also promotes the expression of the inhibitory receptors PD-1, CD160, 2B4 and Lag3, thereby inducing an exhausted state (Shin et al., 2009). T-bet sustains virus-specific $CD8^+$ T cell responses during chronic infection and suppresses the expression of PD-1. As T-bet expression decreases over time during chronic infection PD-1 levels and $CD8^+$ T cell dysfunction both increase (Kao et al., 2011). Eomes also sustains virus-specific $CD8^+$ T cell responses during chronic infection, but its expression is also associated with a more terminally exhausted state with high PD-1 expression levels, indicating Eomes also plays a role in promoting exhaustion (Doering

et al., 2012; Paley et al., 2012). Thus, the same transcription factors responsible for driving effector $CD8^+$ T cell differentiation and memory formation during acute infection are also responsible for limiting responses and preventing immunopathology in the context of chronic infection. Any attempt to manipulate these factors for purposes of immunotherapy will have to take these dual roles into account so that potential functional gains are not negated by simultaneously increasing terminal differentiation and exhaustion.

Transcriptional regulation of human $CD8^+$ T cells

Although the molecules described above have been studied extensively in mouse models, much less is known about them in the context of human immunology. Investigation of transcription factors in human lymphocytes at the single cell level is largely limited to T-bet and Eomes as poor reagent availability precludes the assessment of other transcription factors at this time. In the first report to fully characterize the expression of T-bet and Eomes in healthy human T cells, we found that Eomes is typically expressed with T-bet in $CD8^+$ T cells and, in most cases, is bimodally distributed (McLane et al., 2013). T-bet, on the other hand, has a distinct trimodal expression pattern with a clear intermediate (or $T\text{-bet}^{Lo}$) population in the majority of individuals. We also found that differential T-bet expression levels are associated with specific functional characteristics: $T\text{-bet}^{Lo}$ and negative cells are more likely to express IL-2 compared to $T\text{-bet}^{Hi}$ cells, which tend to express perforin and granzyme B (Makedonas et al., 2010). This data is in agreement with data from mouse models in which T-bet promotes perforin and granzyme B expression while repressing IL-2 production. T-bet and Eomes expression patterns also

closely associate with memory phenotype (McLane et al., 2013; van Aalderen et al., 2015) [**Fig. 1**]: naïve cells express little to no T-bet; T_{CM} cells express very low levels of both; there are more T_{EM} cells expressing T-bet overall, but with low MFI; and T_E cells express high levels of T-bet, with a large population of cells having high T-bet MFI. Eomes association with memory phenotype in healthy humans diverges somewhat from the mouse model, although there may be instances in human infections where these may be more similar (Paley et al., 2012). We found that significantly more cells in the T_E and T_{EM} subsets express Eomes than in the T_{CM} subset and, in the case of T_{EM} cells, Eomes is also expressed at a higher MFI. Polychromatic imaging cytometry (Amnis Imagestream^X) analysis of the T-bet^{Hi} and T-bet^{Lo} cells revealed an important difference in the localization of T-bet in resting human T cells; nuclear localization was generally observed in T-bet^{Hi} cells cytoplasmic localization was more common in T-bet^{Lo} cells (McLane et al., 2013). Looking at T-bet expression levels and localization in memory populations, T_{CM} cells generally have low levels of T-bet that is in the cytoplasm whereas T_E cells tend to express higher levels of T-bet which is mostly nuclear. Thus while a cell may contain T-bet, if it is not expressed at high enough levels it remains sequestered in the cytoplasm of the cell and is transcriptionally inactive.

T-bet expression by HIV-specific CD8$^+$ T cells

Having previously shown that HIV-specific CD8$^+$ T cells from chronic progressors have a reduced ability to upregulate perforin compared to ECs, we examined the potential role

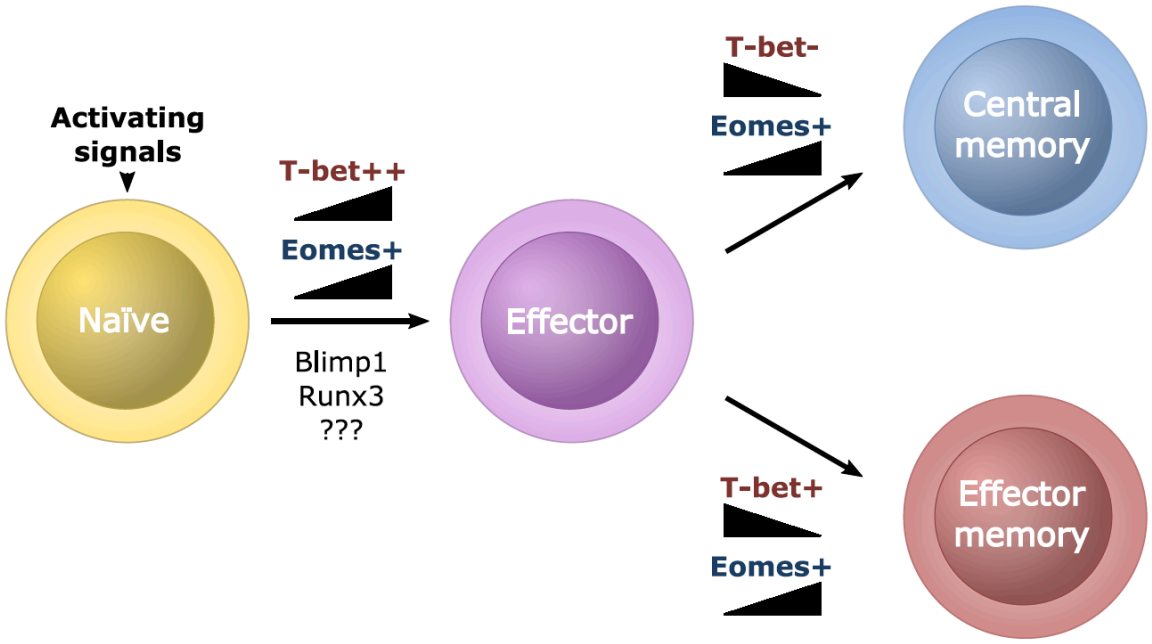

Figure 1. Linear model of CD8+ T cell differentiation with relative expression levels of T-bet and Eomes at each stage. Following antigen-specific activation of a naïve $CD8^+$ T cell, both T-bet and Eomes expression increase, driving effector differentiation. Fully effector differentiated $CD8^+$ T cells have higher levels of T-bet relative to Eomes and to other differentiation states. Lower levels of T-bet relative to Eomes allow generation of memory $CD8^+$ T cells. Most effector memory $CD8^+$ T cells express T-bet but at lower levels relative to effector cells whereas central memory $CD8^+$ T cells express little or no T-bet. Whether changes in Eomes levels are necessary or changes in the ratio of T-bet:Eomes due to the decreased T-bet expression is sufficient to achieve the different differentiation states is not clear. It is also not clear what the roles of other transcription factors such as Blimp1 and Runx3 might be in determining differentiation state. It should be noted that this represents one of several possible models, including a non-linear model in which all differentiation states arise directly from the naïve cell due to differential localization of the transcription factors to progeny during cell division.

of T-bet in driving effector function in each of these two groups (Hersperger et al., 2011). Similar to the expression pattern observed in normal healthy donors, perforin and granzyme B were closely associated with T-bet^{Hi} CD8^{+} T cells in the context of HIV infection. More importantly, we demonstrated that T-bet is significantly lower in HIV-specific CD8^{+} T cells from CPs compared to ECs. Recent reports from Ribeiro-Dos-Santos *et al.* and Buggert *et al.* confirmed this finding, showing reduced T-bet mRNA and protein production during chronic HIV infection (Buggert et al., 2014; Ribeiro-dos-Santos et al., 2012).

Conclusions and Thesis Goals

The identification of immune correlate(s) of protection will help form the basis on which to engineer and assess HIV vaccines and cure strategies. The ability of HIV-specific CD8^{+} T cells to clear infected cells through cytotoxic mechanisms represents the strongest indication of protective potential to date. However, HIV-specific CD8^{+} T cells from most chronically infected individuals demonstrate diminished ability to upregulate the key cytolytic molecule, perforin. Understanding if, when, and why effector CD8^{+} T cell responses are lost over the course of HIV infection could provide insight for strategies that aim to induce or maintain this function. As such, the primary goal of this project was to assess the dynamics of perforin expression in *ex vivo* HIV-specific CD8^{+} over the course HIV infection. A secondary aim was to determine the relationship between perforin expression and the transcription factors T-bet and Eomes, during both acute infection and in chronically infected individuals who differentially control viral replication in the absence of therapy.

CHAPTER 2

TEMPORAL DYNAMICS OF CD8$^+$ T CELL EFFECTOR RESPONSES DURING PRIMARY HIV INFECTION

Summary

The loss of HIV-specific CD8$^+$ T cell cytolytic function is a primary factor underlying progressive HIV infection, but whether HIV-specific CD8$^+$ T cells initially possess cytolytic effector capacity, and when and why this may be lost during infection, is unclear. Here, we assessed CD8$^+$ T cell functional evolution from primary to chronic HIV infection. We observed a profound expansion of perforin$^+$ CD8$^+$ T cells immediately following HIV infection that quickly waned after acute viremia resolution. Selective expression of the effector-associated transcription factors T-bet and eomesodermin in cytokine-producing HIV-specific CD8$^+$ T cells differentiated HIV-specific from bulk memory CD8$^+$ T cell effector expansion. As infection progressed expression of perforin was maintained in HIV-specific CD8$^+$ T cells with high levels of T-bet, but not necessarily in the population of T-bet^{Lo} HIV-specific CD8$^+$ T cells that expand as infection progresses. Together, these data demonstrate that while HIV-specific CD8$^+$ T cells in acute HIV infection initially possess cytolytic potential, progressive transcriptional dysregulation leads to the reduced CD8$^+$ T cell perforin expression characteristic of chronic HIV infection.

Introduction

$CD8^+$ T cells play a central role in the control of HIV replication. During acute infection the emergence of HIV-specific $CD8^+$ T cells correlates with resolution of peak viremia (Borrow et al., 1994; Koup et al., 1994), and in the nonhuman primate model experimental depletion of $CD8^+$ T cells prior to infection with simian immunodeficiency virus delays resolution of acute viremia until the $CD8^+$ T cell pool is reconstituted (Schmitz et al., 1999). Further evidence of the immunologic pressure exerted by $CD8^+$ T cells is manifest by CTL escape mutations throughout all phases of HIV infection and the association of certain MHC class I alleles with superior control of viral replication (Borrow et al., 1997; Carrington and O'Brien, 2003; Fischer et al., 2010; Goonetilleke et al., 2009a; Migueles et al., 2000; Price et al., 1997). However, for the vast majority of infected individuals control is incomplete and ultimately fails in the absence of therapy. A better understanding of the $CD8^+$ T cell response to HIV may inform the design of vaccines, therapeutics, or eradication strategies designed to stimulate or potentiate the natural response to infection resulting in better, if not complete, control.

The $CD8^+$ T cell response to viral infection is multifaceted, including the ability to proliferate, produce multiple cytokines and chemokines, degranulate, and induce cytolysis upon contact with infected targets (Demers et al., 2013). During chronic progressive infection, HIV-specific $CD8^+$ T cells have impaired proliferative potential (Migueles et al., 2002; Migueles et al., 2009; Zimmerli et al., 2005), are less capable of multifunctional responses (Almeida et al., 2007; Betts et al., 2006), and have reduced cytotoxic capacity (Appay et al., 2000; Chen et al., 2009; Hersperger et al., 2011; Migueles et al., 2008; Saez-Cirion et al., 2007). The primary mechanism by which $CD8^+$

T cells kill virally infected cells is via exocytosis of granules containing the cytolytic proteins perforin and granzyme B (Barry and Bleackley, 2002; Podack, 1989). Control of HIV viremia has been associated with the ability of $CD8^+$ T cells from chronically HIV-infected donors to upregulate these cytotoxic effector molecules following *in vitro* culture (Migueles et al., 2008), and we have shown that $CD8^+$ T cell cytotoxic potential, defined by the ability to rapidly upregulate perforin following brief stimulation *ex vivo,* correlates inversely with viral load (Hersperger et al., 2010).

Effector $CD8^+$ T cell development is coordinated by an array of transcription factors (Kaech and Cui, 2012). Murine studies have identified the T-box transcription family members T-bet and eomesodermin (Eomes) as important regulators of the differentiation and function of cytotoxic effector T cells (Cruz-Guilloty et al., 2009; Pearce et al., 2003; Sullivan et al., 2003). T-bet positively regulates genes associated with effector functions including perforin, granzyme B, and IFN-γ (Hersperger et al., 2011; Jenner et al., 2009), whereas Eomes is associated with the expression of perforin as well as proteins involved in maintenance of memory $CD8^+$ T cells (Banerjee et al., 2010; Cruz-Guilloty et al., 2009; Joshi et al., 2007; Pearce et al., 2003). While previous studies suggested a level of redundancy in the gene targets of these transcription factors, recent data show that the balance of T-bet and Eomes expression within a cell is a determinant of the differentiation pathway and functionality of the cell (Buggert et al., 2014; Joshi et al., 2007; McLane et al., 2013; Paley et al., 2012; van Aalderen et al., 2015). In the context of chronic HIV infection, HIV-specific $CD8^+$ T cells with high levels of T-bet demonstrate greater overall functionality and maintain the ability to express perforin whereas cells with a T-$bet^{Lo}$$Eomes^{Hi}$ phenotype are less differentiated, less functional,

exhausted, and express little to no perforin (Buggert et al., 2014; Hersperger et al., 2011). Notably, during chronic progressive infection the T-bet^{Lo}Eomes^{Hi} phenotype dominates the HIV-specific CD8^{+} T cell pool (Buggert et al., 2014). It remains unclear if low T-bet levels and the associated deficiency in perforin expression results from progressive loss on the part of responding HIV-specific CD8^{+} T cells or if responding cells are inherently dysfunctional throughout the infection period.

Much of our current knowledge regarding the dynamics of CD8^{+} T cell responses during acute infection is derived from murine models, particularly following infection with lymphocytic choriomeningitis virus, gammaherpesvirus, or influenza (Butz and Bevan, 1998; Doherty and Christensen, 2000; Murali-Krishna et al., 1998). Infection by these viruses induces rapid and substantial activation and expansion of antigen-specific CD8^{+} T cells. Following resolution of acute viremia, the virus-specific population contracts, giving rise to memory cells that provide long-term protection. Human antiviral CD8^{+} T cell responses have primarily been assessed in the context of chronic infection, after the memory pool has been established (Appay et al., 2000; Catalina et al., 2002; Demers et al., 2013; Lechner et al., 2000; Urbani et al., 2002). Recent studies have examined development of human CD8^{+} T cell responses to a range of primary infections, including attenuated yellow fever virus, attenuated vaccinia virus, influenza, tick-borne encephalitis virus (TBEV), hantavirus, and Epstein-Barr virus (Blom et al., 2015; Lindgren et al., 2011; Miller et al., 2008; Odumade et al., 2012; Precopio et al., 2007; Wilkinson et al., 2012), demonstrating that antigen-specific cells have immediate cytotoxic capacity directly *ex vivo* during the acute phase of these infections. The few studies to examine the earliest responses to HIV showed that HIV-specific CD8^{+} T cells

have limited functionality during the acute phase of infection but did not assess cytotoxic potential or regulation by T-bet or Eomes (Ferrari et al., 2011; Ndhlovu et al., 2015), leaving the question unresolved as to whether these effector molecules are induced during acute infection.

Here, we examined the temporal dynamics of the $CD8^+$ T cell effector response in peripheral blood of subjects experiencing acute primary HIV infection. We found that infection elicited a robust and highly activated response with immediate cytotoxic potential within the peripheral $CD8^+$ T cell pool and that cells responding to short *in vitro* stimulation with HIV peptides were able to degranulate and rapidly upregulate perforin *de novo*. However, HIV-specific $CD8^+$ T cells rapidly lost the ability to upregulate perforin following resolution of peak viremia. Loss of perforin expression coincided with a concurrent reduction in the expression of T-bet, but not Eomes, on a per-cell basis. Our data provide evidence of a robust and physiologically appropriate response during the earliest phase of acute HIV infection that is rapidly lost during progressive chronic infection, due in part to an inability to express sufficient levels of T-bet to properly drive effector differentiation.

Materials and Methods

Study participants: Eleven HIV-1 acutely infected participants were enrolled as part of the RV217 Early Capture HIV cohort (Dr. Merlin Robb, Military HIV Research Program), nine were enrolled in the CHAVI 001 acute infection cohort (Dr. Barton Haynes, Center for HIV/AIDS Vaccine Immunology), and twelve were enrolled in the Montreal Primary Infection cohort (Drs. Nicole Bernard Jean-Pierre Routy, McGill

University). Participant demographics are summarized in S1 Table. Acute HIV-1 infection was determined by measuring plasma HIV RNA content and HIV-specific antibodies using ELISA and Western blot. Fiebig staging (Fiebig et al., 2003) immediately following the first positive visit or at the screening visit was used to characterize the timing of infection for RV217 and CHAVI participants, respectively. The only exception was RV217 donor 40067 for which the estimated date of infection was taken as the midpoint between the last negative and first positive visit. For the Montreal Primary Infection cohort the following guidelines proposed by the Acute HIV Infection Early Disease Research Program sponsored by the National Institutes of Health were used to estimate the date of infection: the date of a positive HIV RNA test or p24 antigen assay available on the same day as a negative HIV enzyme immunoassay (EIA) test minus 14 days; or the date of the first intermediate Western blot minus 35 days. In addition, information obtained from questionnaires addressing the timing of high-risk behavior for HIV transmission was taken into account in assigning a date of infection when consistent with biological tests. The timing of visits relative to estimated date of infection for all acutely HIV infected donors used in this study is provided in Fig 1a. Study participants were antiretroviral therapy naïve at all time points analyzed, consistent with the standard of care at the time of study. HIV-1 viral loads were measured using the Abbot Real-Time HIV-1 assay (RV217; Abbot Laboratories, Abbott Park, IL), COBAS AMPLICOR HIV-1 monitor test, version 1.5 (CHAVI; Roche Diagnostics, Branchburg, NJ), or the UltraDirect Monitor assay (Montreal; Roche Diagnostics, Branchburg, NJ). HIV set point viral loads were defined as the average of all viral load measurements

between 90 and 365 days post-infection in the absence of therapy with the requirement for at least two viral load measurements during this period.

For HIV-negative cohorts, volunteers were administered the live-attenuated YFV-17D vaccine (YF-Vax, Sanofi Pasteur), the live vaccinia smallpox vaccine (Dryvax, Wyeth Laboratories), or challenged with influenza A/Brisbane/59/07. YF-Vax was administered subcutaneously in the arm, Dryvax was administered by scarification of the upper arm with three pricks of a bifurcated needle, and influenza A virus was administered intra-nasally. Peripheral blood mononuclear cells (PBMCs) from pre-vaccination or pre-infection time points were available for most donors along with several time points post-vaccination or infection. PBMC for YFV-vaccinated donors were provided by Dr. Mark Slifka (Orgeon Health and Science University) or Drs. Rafi Ahmed and Rama Akondy (Emory University); vaccinia-vaccinated donor samples were provided by Dr. Mark Slifka (Orgeon Health and Science University); influenza-infected donor samples were provided by Dr. Andrew McMichael (Oxford University). Pre-infection time points from all cohorts, including RV217 participants, along with PBMCs obtained from fifteen healthy human subjects through the University of Pennsylvania's Human Immunology Core were combined for a total of 41 healthy donor data points.

Peptides: Potential T cell epitope (PTE) peptides corresponding to the HIV-1 Gag and Nef proteins were obtained from the NIH AIDS Reagent Program (NIH, Bethesda, Maryland, USA). PTE peptides are 15 amino acids in length and contain naturally occurring 9 amino acid sequences that are potential T cell determinants embedded in the sequences of circulating HIV-1 strains worldwide, including subtypes A, B, C, D and

circulating recombinant forms (CRF). As such, these peptide pools provided the coverage necessary for the T cell stimulation assays performed in this study given the broad geographical distribution of our study participants and diversity of infecting viruses (S1 Table). Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis/Missouri, USA), combined into two pools at 400 μg/ml, and stored at -20°C.

PBMC stimulation: Cryopreserved PBMCs were thawed and rested overnight at $2x10^6$ cells/ml in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cell viability was checked both immediately after thawing and after overnight rest by trypan blue exclusion. Costimulatory antibodies (anti-CD28 and anti-CD49d, 1 μg/mL each; BD Biosciences) and pre-titrated fluorophore conjugated anti-CD107a was included at the start of all stimulations. PBMCs were incubated for 1 hour at 37°C and 5% CO_2 prior to the addition of monensin (1 μg/mL; BD Biosciences) and brefeldin A (10 μg/mL; Sigma-Aldrich) followed by an additional 5 hour incubation at 37°C and 5% CO_2. For peptide stimulations, peptides from the two Gag PTE pools were added to a single tube of cells such that each individual peptide was at a final concentration of 1 μg/ml. As a negative control, DMSO was added to the cells at an equivalent concentration to the one used for peptide stimulation.

Antibody reagents: Antibodies for surface staining included CCR7 APC-Cy7 (clone

G043H7; Biolegend), CCR7 APC-eFluor780 (clone 3D12; eBioscience), CD4 PE-Cy5.5 (clone S3.5; Invitrogen), CD8 BV711 (clone RPA-T8; Biolegend), CD8 Qdot 605 (clone 3B5; Invitrogen), CD14 BV510 (clone M5E2; Biolegend), CD14 Pacific Blue (clone M5E2; custom), CD14 PE-Cy5 (clone 61D3; Abcam), CD14 PE-Cy7 (clone HCD14; Biolegend), CD16 Pacific Blue (clone 3G8; custom), CD16 PE-Cy5 (clone 3G8; Biolegend), CD16 PE-Cy7 (clone 3G8; Biolegend), CD19 BV510 (clone HIB19; Biolegend), CD19 Pacific Blue (clone HIB19; custom), CD19 PE-Cy5 (clone HIB19; Biolegend), CD19 PE-Cy7 (clone HIB19; Invitrogen), CD45RO ECD (clone UCHL1; Beckman Coulter), CD45RO PE-CF594 (clone UCHL1; BD Biosciences), CD107a PE-Cy5 (clone eBioH4A3; eBioscience), CD107a PE-Cy7 (clone H4A3; Biolegend), and HLA-DR Pacific Blue (clone LN3; Invitrogen). Antibodies for intracellular staining included: CD3 BV570 (clone UCHT1; Biolegend), CD3 BV650 (clone OKT3; Biolegend), CD3 Qdot 585 (clone OKT3; custom), CD3 Qdot 650 (clone S4.1; Invitrogen), Eomes Alexa 647 (WD1928; eBioscience), Eomes eFluor 660 (WD1928; eBioscience), IFN-γ Alexa 700 (clone B27; Invitrogen), Perforin BV421 (clone B-D48, Biolegend), Perforin Pacific Blue (clone B-D48; custom), Perforin PE (clone B-D48, Cell Sciences), T-bet FITC (clone 4B10; Biolegend), and T-bet PE (clone 4B10; eBioscience).

Flow cytometric analysis: At the end of the stimulations, cells were washed once with PBS prior to be being stained for CCR7 expression for 15 min at 37°C in the dark. Cells were then stained for viability with aqua amine-reactive viability dye (Invitrogen) for 10 min at room temperature in the dark followed by addition of a cocktail of antibodies to stain for surface markers for an additional 20 min. The cells were washed with PBS

containing 0.1% sodium azide and 1% BSA, fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), and stained with a cocktail of antibodies against intracellular markers for 1 h at room temperature in the dark. The cells were washed once with Perm Wash buffer (BD Biosciences) and fixed with PBS containing 1% paraformaldehyde. Fixed cells were stored at 4°C in the dark until acquisition. Antibody capture beads (BD Biosciences) were used to prepare individual compensation controls for each antibody used in the experiment. ArC Amine Reactive beads (ThermoFisher Scientific) were used to generate a singly stained compensation control for the aqua amine-reactive viability dye.

For each stimulation condition, a minimum of 250,000 total events were acquired using a modified LSRII (BD Immunocytometry Systems). Data analysis was performed using FlowJo (TreeStar) software. Gating strategy is provided in **Fig. 2**. Reported antigen-specific data have been corrected for background based on the negative (no peptide) control, and only responses with a total frequency twice the negative control and above 0.01% of total memory $CD8^+$ T cells (after background subtraction) were considered to be positive responses. By analyzing the data in this way, we examined cytolytic protein production resulting from antigen-specific stimulation and ensured that its expression was considered only within responding CD8 T cells expressing at least one other functional parameter. Whereas IFN-γ, CD107a, and MIP-1α were used to identify antigen-specific $CD8^+$ T cells for some donors, only IFN-γ and CD107a were used consistently for all donors and figures depicting antigen-specific data were derived from analysis of cells expressing these two markers unless otherwise noted.

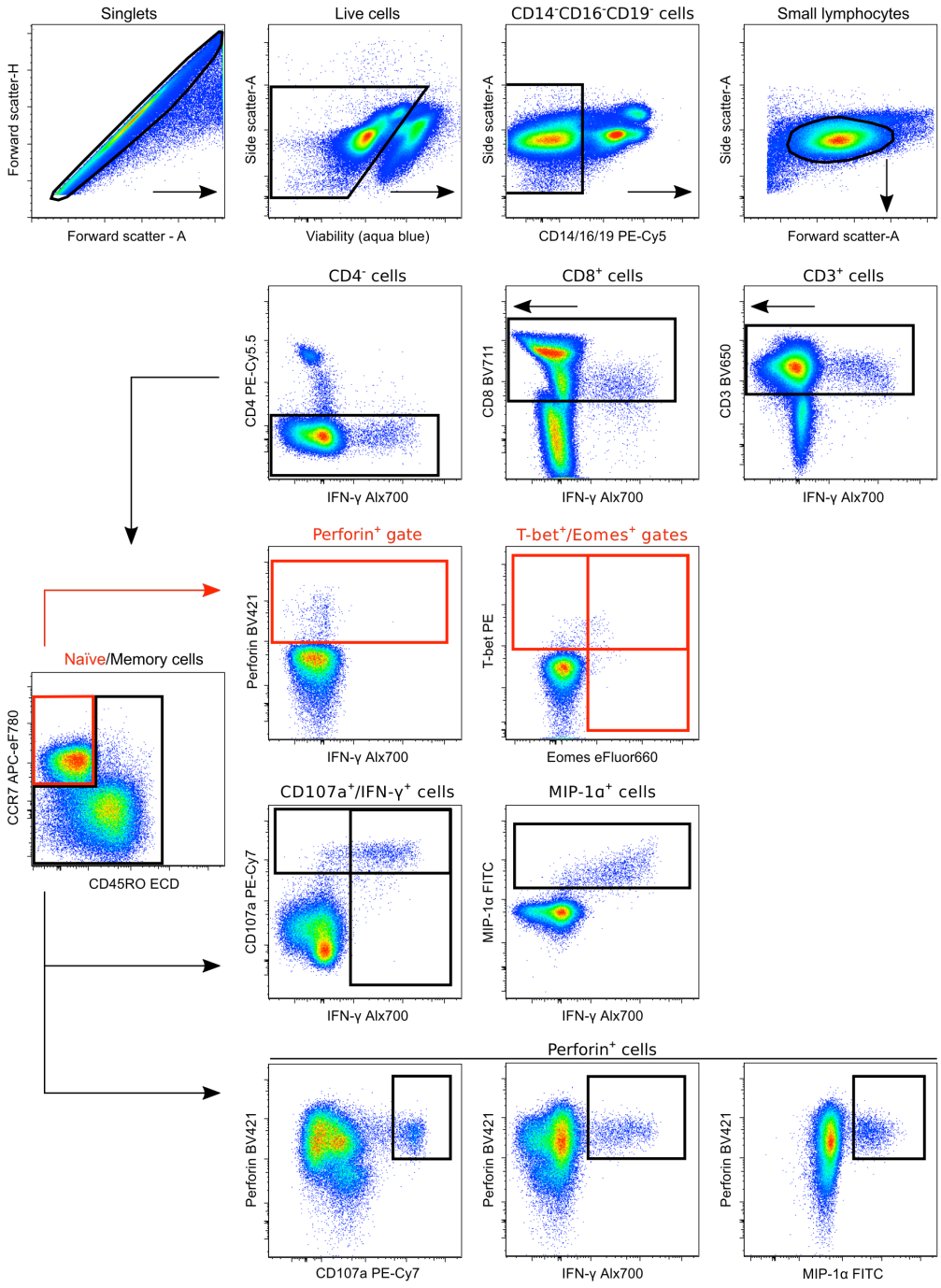

Figure 2. Gating strategy for the polychromatic flow cytometric staining panel. General gating strategy for a representative donor to identify total CD8$^+$ T cells, CCR7 and CD45RO memory subsets, total perforin$^+$ cells, T-bet$^+$ cells, Eomes$^+$ cells, and responding cells (IFN-γ$^+$, CD107a$^+$, or MIP-1α) following stimulation with Gag peptides. Gag-specific cells were assessed to be perforin$^+$ if they expressed perforin in conjunction with IFN-γ, CD107a, or MIP-1α.

Table 1. Acute/early HIV cohort demographics

Study[a]	Donor ID	Gender	Age	Country	Days Pre-infection[b]	Days Infected[b,c]	RNA copies/ml[c]	Clade
CHAVI	700010621	M	40	USA	-	125	387344	N.D.
CHAVI	700010717	F	30	USA	-	29	750000	N.D.
CHAVI	701010248	M	24	USA	-	29	2485	N.D.
CHAVI	702010157	M	21	Malawi	-	29	750000	N.D.
CHAVI	702010176	M	21	Malawi	-	67	750000	N.D.
CHAVI	702010202	M	30	Malawi	-	293	64114	N.D.
CHAVI	702010280	M	21	Malawi	-	125	295688	N.D.
CHAVI	703010200	M	24	Malawi	-	38	125291	C
CHAVI	703010217	F	34	Malawi	-	29	102602	C
Montreal	ACT90233	M	31	Canada	-	68	11886	B
Montreal	ACT92900	M	31	Canada	-	61	8392	B
Montreal	GOL033G	M	29	Canada	-	77	94436	B
Montreal	GOL036G	M	59	Canada	-	71	181711	B
Montreal	GOL037G	M	41	Canada	-	60	60799	B
Montreal	GOL038G	M	44	Canada	-	55	4889	B
Montreal	HNDDRPI050	M	35	Canada	-	177	11785	B
Montreal	HNDDRPI060	M	32	Canada	-	174	87768	B
Montreal	HNDDRPI062	M	34	Canada	-	79	207458	B
Montreal	HNDDRPI063	M	55	Canada	-	52	108207	B
Montreal	HNDDRPI064	M	27	Canada	-	70	87887	B
Montreal	HNDDRPI070	M	33	Canada	-	61	145228	B
RV217	10220	F	33	Uganda	-197	31	309030	A1
RV217	10374	F	26	Uganda	-174	41	10965	A1D
RV217	20225	F	24	Kenya	-125	24	1122018	A1C
RV217	30507	F	34	Tanzania	-277	29	1122	B/CRF01
RV217	40067	M	27	Thailand	-441	27	229087	A1C
RV217	40094	M	19	Thailand	-237	29	2511886	CRF01_AE
RV217	40123	M	23	Thailand	-238	31	630957	CRF01_AE
RV217	40134	M	18	Thailand	-48	23	2454708	C/CRF01
RV217	40250	M	35	Thailand	-159	28	4677351	CRF01_AE
RV217	40283	M	23	Thailand	-456	23	398107	B
RV217	40353	M	21	Thailand	-27	28	162181	B

[a] CHAVI = Center for HIV/AIDS Vaccine Immunology, Montreal = McGill University Health Center, RV217 = US Military HIV Research Program.

[b] Estimated days from infection was based on Fiebig staging at first positive visit (CHAVI and RV217) or available serological parameters in combination with donor proposed date (Montreal).

[c] At first time point analyzed.

Statistical analysis: All statistical analysis was performed using Stata (version 14.0). Graphs were generated using Stata or GraphPad Prism (version 5.0a). Generalized estimating equations (GEEs) with robust variances were used to test for changes while adjusting for repeated measurements on the same individuals (Liang and Zeger, 1986). In instances where many values were at 100% a random-effects tobit regression model was used to do a combined analysis of the percent of data points at 100% versus differences in values for data points below 100%. *P* values were Holm-adjusted for multiple comparisons. Bars represent approximations of the means generated by the models. Lowess smoothers were used to represent the mean over time for longitudinal data. Correlations were determined using Spearman's rank correlation test (non-parametric; two-tailed).

Results

Acute HIV infection is associated with an expansion of the effector memory $CD8^+$ T cell pool

Longitudinal samples were obtained from 32 subjects experiencing primary HIV infection (**Fig. 3A**), 28 of whom had at least one acute time point (36 time points total; median 54 d from infection, range 23-100 d) and 23 with at least one chronic time point (40 time points total; median 551 d, range 367-880 d). Samples were drawn from three separate cohorts of acutely infected individuals: the CHAVI001 acute-infection cohort, the Montreal Primary Infection cohort, and the RV217/ECHO cohort. These cohorts provided broad geographical representation including North America, East Africa,

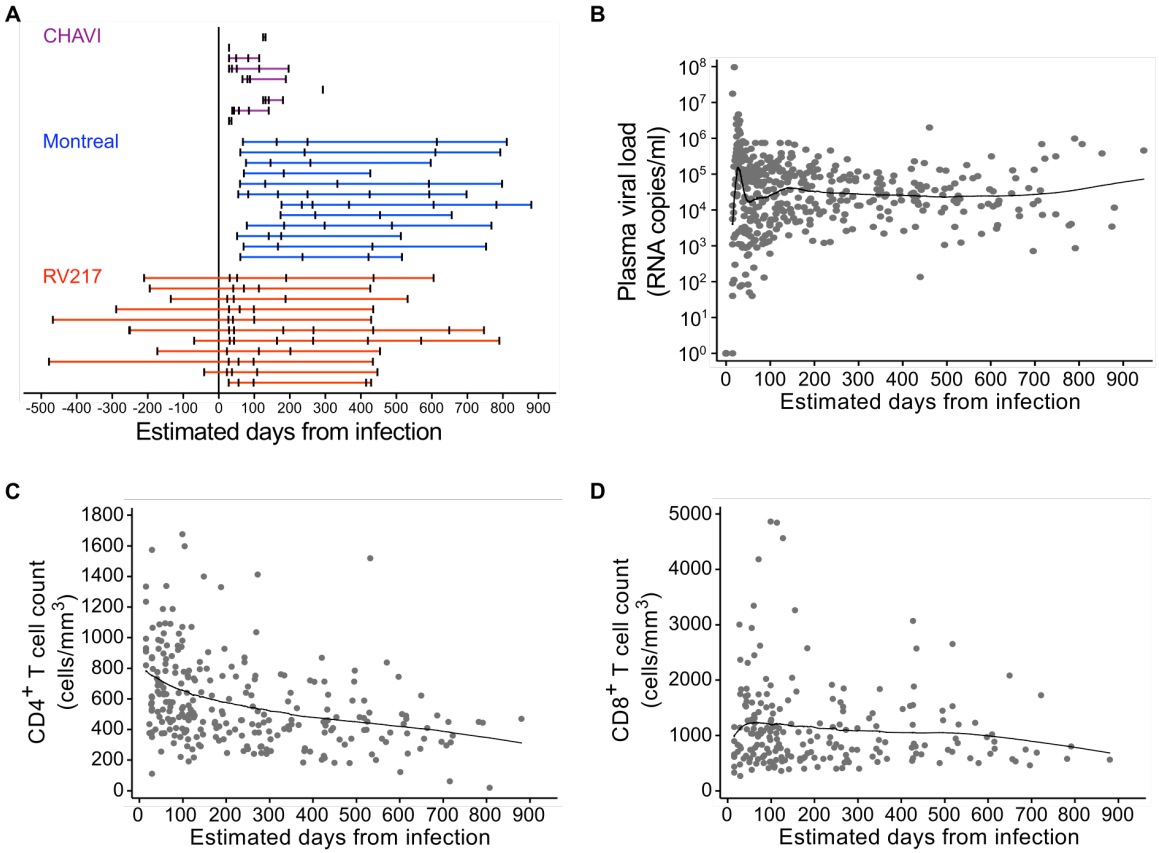

Figure 3. Timing of study participant samples and dynamics of HIV plasma viral loads, CD4$^+$ T cell counts and CD8$^+$ T cell counts. (**A**) Timing of samples relative to estimated time of infection for the three acute/early HIV cohorts: CHAVI (purple), Montreal (blue), and RV217 (red). Plasma HIV RNA copies/ml (**B**), absolute CD4$^+$ T cells counts (**C**), and CD8$^+$ T cell counts for all 32 donors (**D**). Lowess smoothers were used to represent the mean over time for longitudinal data.

Malawi, and Thailand (Table 1). Subjects were antiretroviral therapy naïve at all time points, consistent with the standard of care at the time of study, and none controlled viral load to undetectable levels (**Fig. 3B**). The mean peak viral load was 5.2 $\log_{10}$ RNA copies/ml for the entire study population (7.0 $\log_{10}$ RNA copies/ml for the better-characterized RV217 donors) and 4.42 $\log_{10}$ RNA copies/ml at set point. Peripheral blood $CD4^+$ T cell counts and $CD8^+$ T cell counts both declined over the study period (average rates of 80 cells/mm^3 per year and 75 cells/mm^3, respectively; **Fig. 3C and 3D**). Samples from 41 seronegative healthy donors, including pre-infection time points for the 11 RV217 acute subjects (median -210 d from infection, range -41 to -478 d; **Fig. 3A and Table 1**), were analyzed for comparison.

To determine if different phases of infection were associated with changes in circulating $CD8^+$ T cell differentiation and activation, we assessed the size and composition of the memory $CD8^+$ T cell pool (**Fig. 4**). Relative to HIV-negative donors, HIV-infected subjects had a significantly larger memory (non-$CCR7^+CD45RO^-$) $CD8^+$ T cell pool in both the acute and chronic phases of infection (**Fig. 4A and 4B**). Of note, the frequency of total memory $CD8^+$ T cells at the earliest post-infection time points inversely correlated with peak viral load, but not with set point viral load (**Fig. 4C** and data not shown). In addition to the larger memory pool we also observed a shift in the distribution of memory subsets in infected subjects, with significantly higher proportions of central memory ($CCR7^+CD45RO^+$) and, predominately, effector memory ($CCR7^-CD45RO^-$) subsets during acute infection (**Fig. 4D**). Only the effector memory pool remained significantly elevated into the chronic phase. There was no difference in the

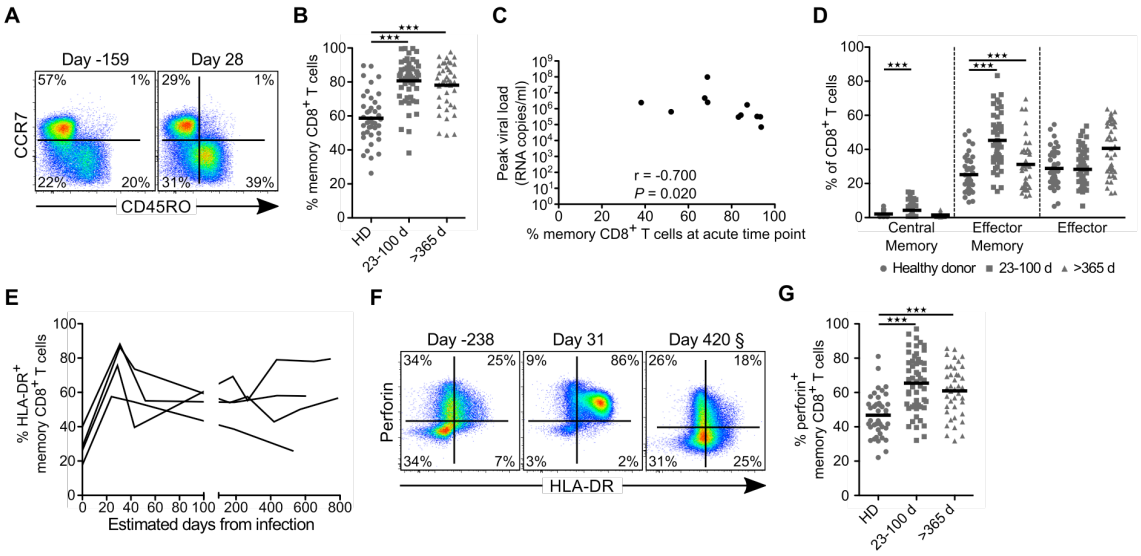

Figure 4. Memory distributions, activation, and proportion of cytotoxic peripheral CD8$^+$ T cells for healthy donors and following HIV infection. (**A**) Representative flow cytometric plots of CCR7 versus CD45RO from a pre- (day -159) and acute (day 28) infection time point for one donor. (**B**) Proportion of circulating total memory CD8$^+$ T cells for all healthy donors (HD), acute HIV time points (23-100 d), and chronic HIV time points (>365 d). (**C**) Peak viral load plotted against total memory CD8$^+$ T cells at the earliest available time point post-infection (23-41 d) for each RV217 donor. Spearman's rank correlation test was used to determine significance. (**D**) Memory subsets as determined by CCR7 and CD45RO staining for all healthy donors (circles), acute HIV time points (squares), and chronic HIV time points (triangles). (**E**) Proportion of memory CD8$^+$ T cells that express HLA-DR over time from infection for four RV217 subjects. Pre-infection time points were set as day 0 for analysis. (**F**) Representative flow cytometric plots of perforin and HLA-DR expression by memory CD8$^+$ T cells from a pre- (day -238), acute (day 31), and chronic (day 420) infection time point for one donor. § Day 420 sample was acquired and analyzed at a later date than earlier samples resulting in a different gating scheme. For consistency, gates were set using naïve (CCR7$^+$CD45RO$^-$) CD8$^+$ T cells, which generally do not express perforin or HLA-DR. (**G**) Proportion of memory CD8$^+$ T cells that express perforin for healthy donors (HD), acute HIV time points (23-100 days), and chronic HIV time points (> 365 days). All data represent direct *ex vivo* assessment with no *in vitro* stimulation. *** denotes a *P* value < 0.001. Statistics based on a GEE population-averaged model with Holm adjusted *P* value. Bars represent approximations of the means generated by the models.

proportion of the effector cell pool ($CCR7^{-}CD45RO^{-}$) during either phase of infection, although the relative frequency of these cells did appear to be larger as infection progressed (**Fig. 4D**).

When we examined the activation state of the memory pool for four RV217 subjects by measuring surface expression of HLA-DR, we found massive levels of activation within the memory $CD8^{+}$ T cell compartment following HIV infection (**Fig. 4E**), in agreement with recent data from Ndhlovu *et al.* (Ndhlovu et al., 2015). To determine if this population of highly activated cells expressed cytolytic molecules directly *ex vivo* we measured perforin content. We found that almost all HLA-DR^{+} cells expressed perforin during the acute phase (**Fig. 4F**). In addition, we observed a significantly greater proportion of $perforin^{+}$ cells in both acute and chronic phases of infection compared to healthy donors (**Fig. 4G**). There was, however, no significant association between the frequency of $perforin^{+}$ $CD8^{+}$ T cells and viral load at any time point (data not shown). Together, these data show that during acute HIV infection a large proportion of the peripheral $CD8^{+}$ T cell pool is highly activated and primed to exert cytotoxic effector activity but the absolute magnitude of total cytotoxic $CD8^{+}$ T cells does not predict set point viral load.

<u>HIV infection increases the total peripheral cytotoxic $CD8^{+}$ T cell pool</u>

We next examined if the large frequency of cytotoxic $CD8^{+}$ T cells observed during acute HIV infection was consistent across other acute viral infections. We compared the total $CD8^{+}$ T cell responses of subjects from the RV217 cohort with those of HIV-negative individuals who were vaccinated with attenuated vaccinia virus (VV) or attenuated

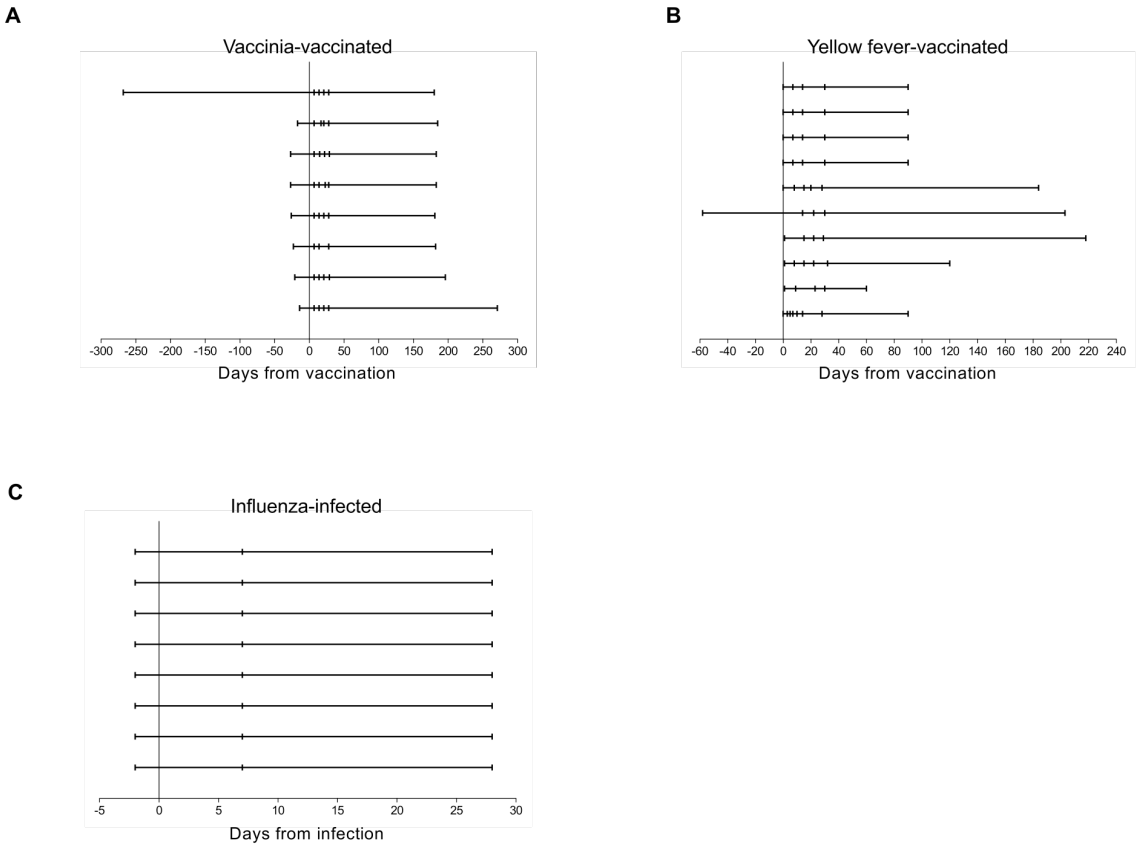

Figure 5. Timing of HIV-seronegative study participant samples. Timing of samples for HIV-seronegative healthy donors relative to vaccination with live attenuated vaccinia virus (**A**), live attenuated yellow fever virus (**B**), or experimental infection with influenza (**C**).

yellow fever virus (YFV)-17D, or experimentally infected with a H1N1 strain of influenza virus (**Fig. 5A-5C**). Vaccination with VV or YFV elicits a robust and highly specific $CD8^+$ T cell response that peaks approximately two weeks after inoculation and is largely resolved by four weeks (Miller et al., 2008). The peripheral $CD8^+$ T cell response to influenza is less robust, peaks at 1-2 weeks, and resolves by four weeks post-infection (Wilkinson et al., 2012).

Consistent with the comparison between healthy donors and acute phase HIV infection (**Fig. 4B**), both the total memory $CD8^+$ T cell pool and the effector memory subset increased significantly from pre- to acute HIV infection (**Fig. 6A** and data not shown). There was also a significant increase in the proportion of $perforin^+$ cells over the first thirty days of infection, with almost all (>90%) circulating memory $CD8^+$ T cells expressing perforin in some donors (**Fig. 6E**). When we examined the $CD8^+$ T cell responses to *in vivo* stimulation following vaccination with VV or YFV, or infection with influenza, we did not observe significant changes in the size or distribution of the peripheral memory pool (**Fig. 6B-6D** and data not shown). We did find increased levels of activated HLA-DR^+ cells in some donors after vaccination with VV and YFV, but frequencies of $perforin^+$ cells remained relatively stable throughout the entire vaccine course (**Fig. 6F and 6G and Fig. 7A-7D**). Only infection with influenza resulted in a slight but significant increase in $perforin^+$ cells at d28 post-infection (**Fig. 6H**). While these models of acute viral infections do have limitations in their use as comparators for our HIV-infected donors (e.g. different antigen loads, different localizations, and more precise timing of infection), overall these data show the dramatic increase in cytotoxic cells that takes place in the peripheral blood of HIV acutely infected subjects is

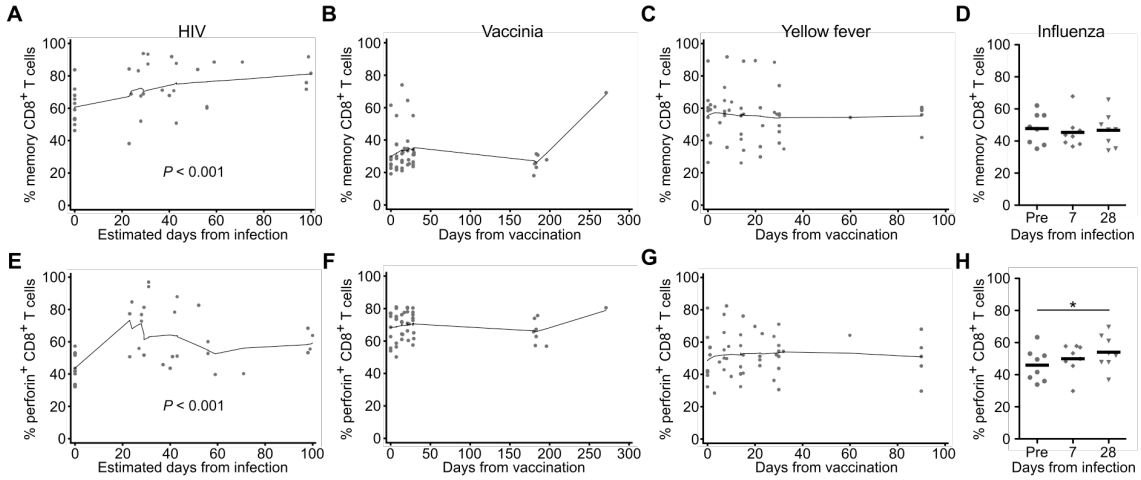

Figure 6. Dynamics of the total CD8+ T cell memory pool and cytotoxic response following infection with HIV, vaccinia virus, yellow fever virus, or influenza. Proportion of total memory $CD8^+$ T cells for longitudinal time points from donors either naturally infected with HIV (**A**), vaccinated with attenuated vaccinia virus (Dryvax; **B**), vaccinated with live yellow fever virus (YFV-17D; **C**), or experimentally infected with influenza (strain H1N1; **D**). Frequency of memory $CD8^+$ T cells that express perforin following infection with HIV (**E**), vaccinia (**F**), yellow fever (**G**) or influenza (**H**). Pre = pre-infection or pre-vaccination time points. Pre-infection time points for HIV, vaccinia, and YFV, were set as day 0 for analysis. All data represent direct *ex vivo* assessment with no *in vitro* stimulation. * denotes a *P* value < 0.05. Statistics based on a GEE population-averaged model with Holm adjusted *P* value. Bars represent approximations of the means generated by the models. Lowess smoothers were used to represent the mean over time for longitudinal data.

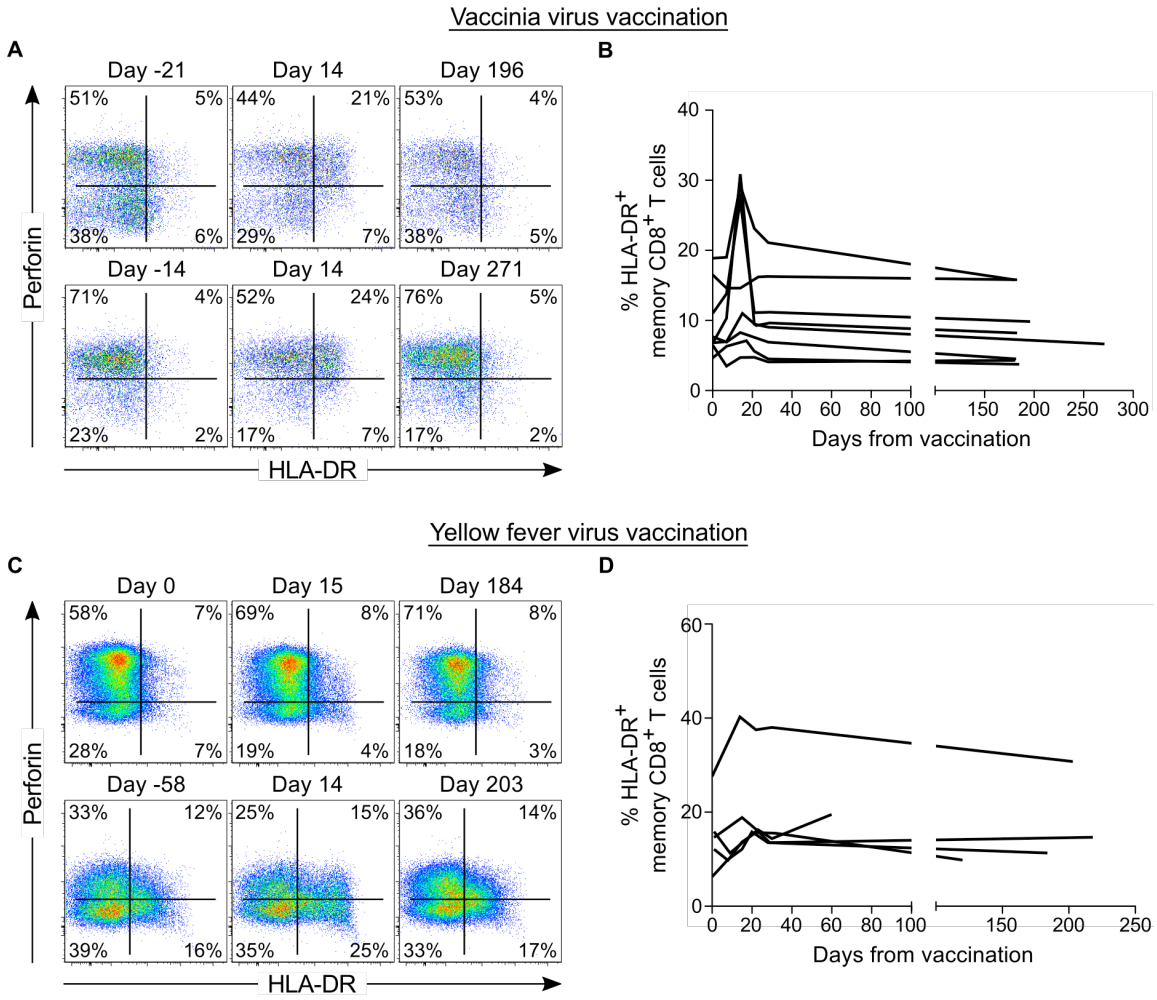

Figure 7. Activation of peripheral memory CD8$^+$ T cells for HIV-negative individuals following vaccination with live attenuated vaccinia virus or live attenuated yellow fever virus. (**A**) Representative flow cytometric plots of perforin versus HLA-DR for two vaccinia-infected subjects. (**B**) Proportion of memory CD8$^+$ T cells that express HLA-DR over time from infection for all vaccinia-vaccinated subjects. (**C**) Representative flow cytometric plots of perforin versus HLA-DR for two yellow fever-vaccinated subjects. (**D**) Proportion of memory CD8$^+$ T cells that express HLA-DR over time from infection for five yellow fever-infected subjects. All data represent direct *ex vivo* assessment with no *in vitro* stimulation.

significantly more pronounced compared to live-attenuated vaccination or influenza infection.

HIV-specific $CD8^+$ T cell cytotoxic capacity decreases as HIV infection progresses

We next sought to determine if the cytotoxic potential of HIV-specific cells demonstrated similar dynamics to the total memory $CD8^+$ T cell pool during acute to chronic HIV infection. To identify HIV-specific cells we focused on the detection of IFN-γ production and CD107a-marked degranulation following a short-term *in vitro* stimulation with peptides derived from the HIV-1 Gag and Nef proteins (Betts et al., 2003; Betts et al., 2006; Ferrari et al., 2011; Ndhlovu et al., 2015). In agreement with previous studies that evaluated HIV-specific cells longitudinally by functional responses or tetramer staining (Ferrari et al., 2011; Trautmann et al., 2012), we found no difference in the absolute magnitude of responding cells for either protein over time (**Fig. 8A** and data not shown). Consistent with the memory distribution of the total $CD8^+$ T cell pool, Gag-specific cells largely had an effector memory phenotype in the acute phase of infection but became more equally distributed between effector and effector memory subsets for early chronic time points (**Fig. 8B and 8C**). Also in agreement with previous data, cells tended to degranulate more readily than upregulate IFN-γ in the acute phase of infection [**Fig. 8D** and data not shown](Ferrari et al., 2011; Ndhlovu et al., 2015). The high proportion of degranulating cells suggested that the HIV-specific response might be cytotoxic over the course of infection, as analysis of the total $CD8^+$ T cell pool had indicated. However, degranulation is not an absolute surrogate of cytolytic potential (Hersperger et al., 2010; Wolint et al., 2004), nor does it indicate whether the cells will continue to be cytotoxic

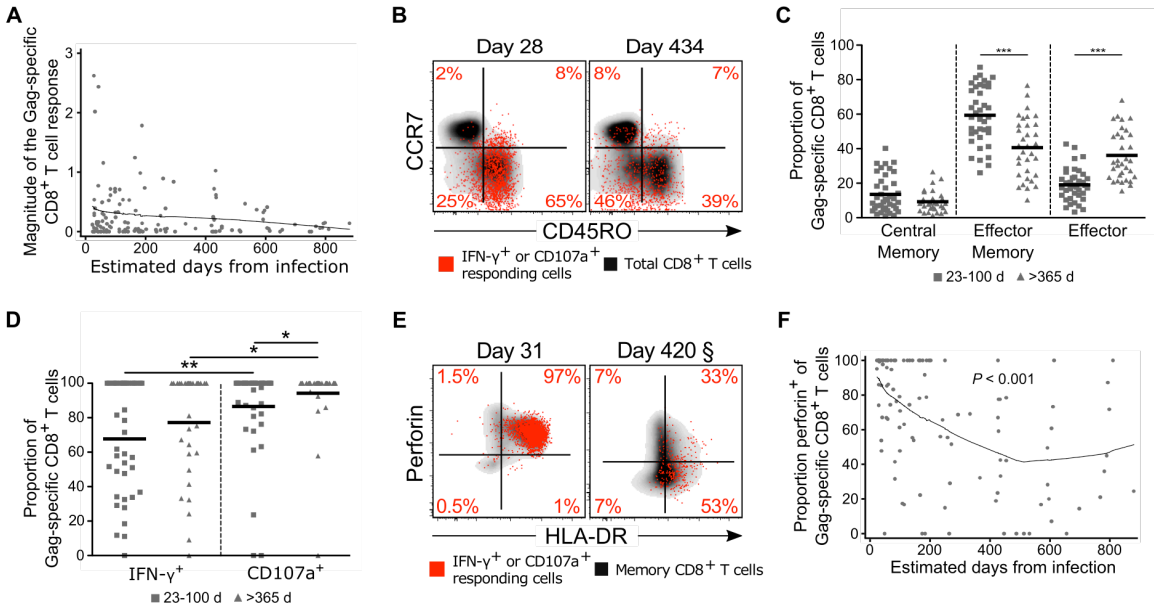

Figure 8. Magnitude and functionality of HIV-specific responses over time. (**A**) Frequency of Gag-specific $CD8^+$ T cells within the memory $CD8^+$ T cell pool over time as determined by measurement of IFN-γ expression or degranulation (CD107a) in response to peptide stimulation. (**B**) Memory distributions for Gag-specific $CD8^+$ T cells (red) overlaid on total $CD8^+$ T cells (black) for a representative donor. (**C**) Memory distributions for responding Gag-specific $CD8^+$ T cells as determined by CCR7 and CD45RO staining for acute (squares), and chronic (triangles) HIV time points. (**D**) Proportion of total responding Gag-specific $CD8^+$ T cells that have upregulated IFN-γ or degranulated at acute and chronic time points. (**E**) Gag-specific $CD8^+$ T cells (red) overlaid on total memory $CD8^+$ T cells (black) for a representative donor. Percentages represent frequency of responding Gag-specific cells within a quadrant. § Day 420 sample was acquired and analyzed at a later date than earlier samples resulting in a different gating scheme. For consistency gates were set using naïve ($CCR7^+CD45RO^-$) $CD8^+$ T cells. (**F**) Proportion of total responding Gag-specific $CD8^+$ T cells that upregulated perforin in response to peptide stimulation ($n = 28$). * denotes a P value < 0.05 and ** denotes a P value < 0.01. Statistics based on a GEE population-averaged model with Holm adjusted P value or random-effects tobit regression. Bars represent approximations of the means generated by the models. Lowess smoothers were used to represent the mean over time for longitudinal data.

following the initial granule release (Makedonas et al., 2009). To assess cytotoxic potential more directly, we measured perforin expression levels within the Gag- and Nef-specific cells (**Fig. 8E** and data not shown). The majority of cells that responded to direct *ex vivo* stimulation rapidly upregulated perforin during the earliest time points following infection, suggesting that the early HIV-specific response was likely highly cytotoxic. In contrast to the bulk memory $CD8^+$ T cell pool, however, as acute viremia was resolved there was a rapid loss of perforin expression by both HIV-1 Gag- and Nef-specific $CD8^+$ T cells (**Fig. 8F** and data not shown).

A large proportion of HIV-specific $CD8^+$ T cells have previously been shown to upregulate β-chemokines independently of degranulation during acute HIV infection (Ferrari et al., 2011). To determine if β-chemokine-producing cells similarly expressed perforin, we assessed expression of MIP-1α by responding cells in a subset of subjects. Inclusion of MIP-1α did not significantly change the overall magnitude of Gag-specific cells detected over time, though it did identify a subset of cells not captured by IFN-γ or CD107a (data not shown). Importantly, the dynamics with which expression of perforin by Gag-specific cells was lost was the same with or without MIP-1α (data not shown). Combined, these data show similarities in the total and Gag-specific $CD8^+$ T cell responses in both differentiation state and cytotoxic potential, suggesting the bulk of activated cells during acute HIV infection could be comprised of HIV-specific $CD8^+$ T cells.

Dissociation of T-bet and Eomes expression from cytolytic potential in memory $CD8^+$ T cells in acute HIV infection

Studies in both murine models and humans have strongly linked the transcription factors T-bet and Eomes to the regulation of effector $CD8^+$ T cell differentiation and function, including the expression of perforin (Blom et al., 2015; Cruz-Guilloty et al., 2009; Hersperger et al., 2011; Joshi et al., 2007; Makedonas et al., 2010; Pearce et al., 2003; Sullivan et al., 2003). To gain further insight into the evolution of the cytotoxic $CD8^+$ T cell response to HIV we assessed the expression of T-bet and Eomes over the course of infection. For healthy donors, including HIV pre-infection time points, perforin expression was directly associated with T-bet and/or Eomes expression such that the majority of perforin$^+$ cells were either T-bet$^+$Eomes$^+$ or T-bet$^+$Eomes$^-$ (**Fig. 9A and 9B**). In contrast, acutely HIV-infected individuals showed marked dissociation between perforin and both T-bet and Eomes resulting in significantly lower proportions of T-bet$^+$Eomes$^+$ and T-bet$^+$Eomes$^-$ perforin$^+$ cells (**Fig. 9A and 9B**), and an expansion of perforin$^+$ cells expressing neither T-bet nor Eomes. By the chronic stage these subsets had largely, though incompletely, returned to their normal distributions. When we analyzed T-bet and Eomes expression longitudinally for perforin$^+$ $CD8^+$ T cells within the HIV-infected cohort we found the proportion of T-bet$^+$Eomes$^+$ cells decreased over the first 30 days of infection and T-bet$^-$Eomes$^-$ cells increased over the first 60 days before gradually returning to pre-infection levels (**Fig. 9C and 9D**). We have previously shown that the level of T-bet expression within peripheral $CD8^+$ T cells is directly associated with perforin expression, where perforin was found predominantly within T-bet^{Hi} cells (Hersperger et al., 2011). Consistent with those findings, perforin was most

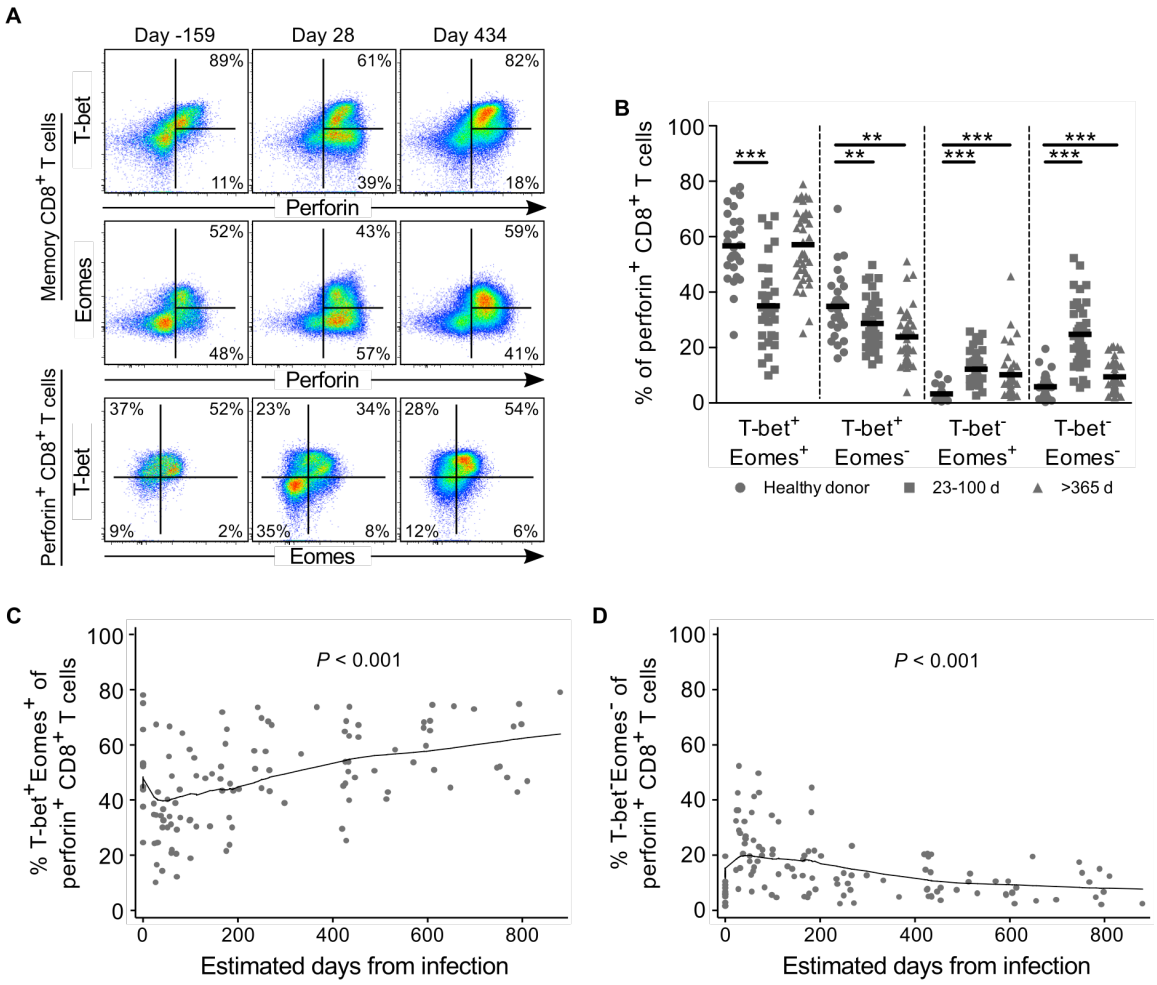

Figure 9. T-bet and Eomes expression by total perforin$^+$ CD8$^+$ T cells for healthy donors and following HIV infection. (**A**) Representative flow cytometric plots of T-bet and Eomes expression for total memory (top and middle rows) and perforin$^+$ (bottom row) CD8$^+$ T cells from the pre- (day -159), acute (day 28), and chronic (day 434) infection time points for one donor. Percentages on top and middle plots are of perforin$^+$ cells within the total memory pool. (**B**) T-bet and Eomes expression by perforin$^+$ CD8$^+$ T cells for all healthy donors (circles), acute HIV time points (squares), and chronic HIV time points (triangles). Frequency of perforin$^+$ cells that are T-bet$^+$Eomes$^+$ (**C**) or T-bet$^-$ Eomes$^-$ (**D**) over time. All data represent direct *ex vivo* assessment with no *in vitro* stimulation. ** denotes a *P* value < 0.01 and *** denotes a *P* value < 0.001. Statistics based on a GEE population-averaged model with Holm adjusted *P* value. Bars represent approximations of the means generated by the models. Lowess smoothers were used to represent the mean over time for longitudinal data.

highly associated with a T-bet^{Hi}Eomes^{+} expression pattern in HIV negative donors and this subset experienced the largest drop during acute HIV (data not shown). Despite these shifts in expression patterns that appeared to coincide with the rise and fall plasma viremia, there was no association between the acute frequencies of T-bet or Eomes subsets and acute or set point viral loads (data not shown). However, frequencies of T-bet^{+} and T-bet^{-}Eomes^{-} CD8^{+} T cells at set point time points were inversely or directly associated with set point viral load, respectively (data not shown).

To determine if the dissociation between perforin, T-bet, and Eomes was unique to HIV, we examined T-bet and Eomes expression within total perforin^{+} cells following YFV and VV vaccination. While we found almost no dissociation for YFV, there was a transient dissociation following vaccination with vaccinia, although not to the same extent as observed during acute HIV (**Fig. 10A and 10B**). We next examined expression of T-bet and Eomes within HLA-DR^{+} cells throughout the different vaccine courses. As noted above, during acute HIV infection the vast majority of HLA-DR^{+} cells are also perforin^{+} (**Fig 4E**); thus, it was unsurprising to find that perforin^{+} and HLA-DR^{+} cells showed almost identical dynamics in the loss of T-bet and Eomes expression for HIV (**Fig. 10C**). Similarly, for both YFV and VV, activated cells showed a transient increase in the frequency of T-bet^{-}Eomes^{-} cells at day 14 post-vaccination. Together these data suggest that the transient expansion of highly activated bulk effector CD8^{+} T cells during acute viral infection in humans may not require expression and/or maintenance of T-bet and Eomes.

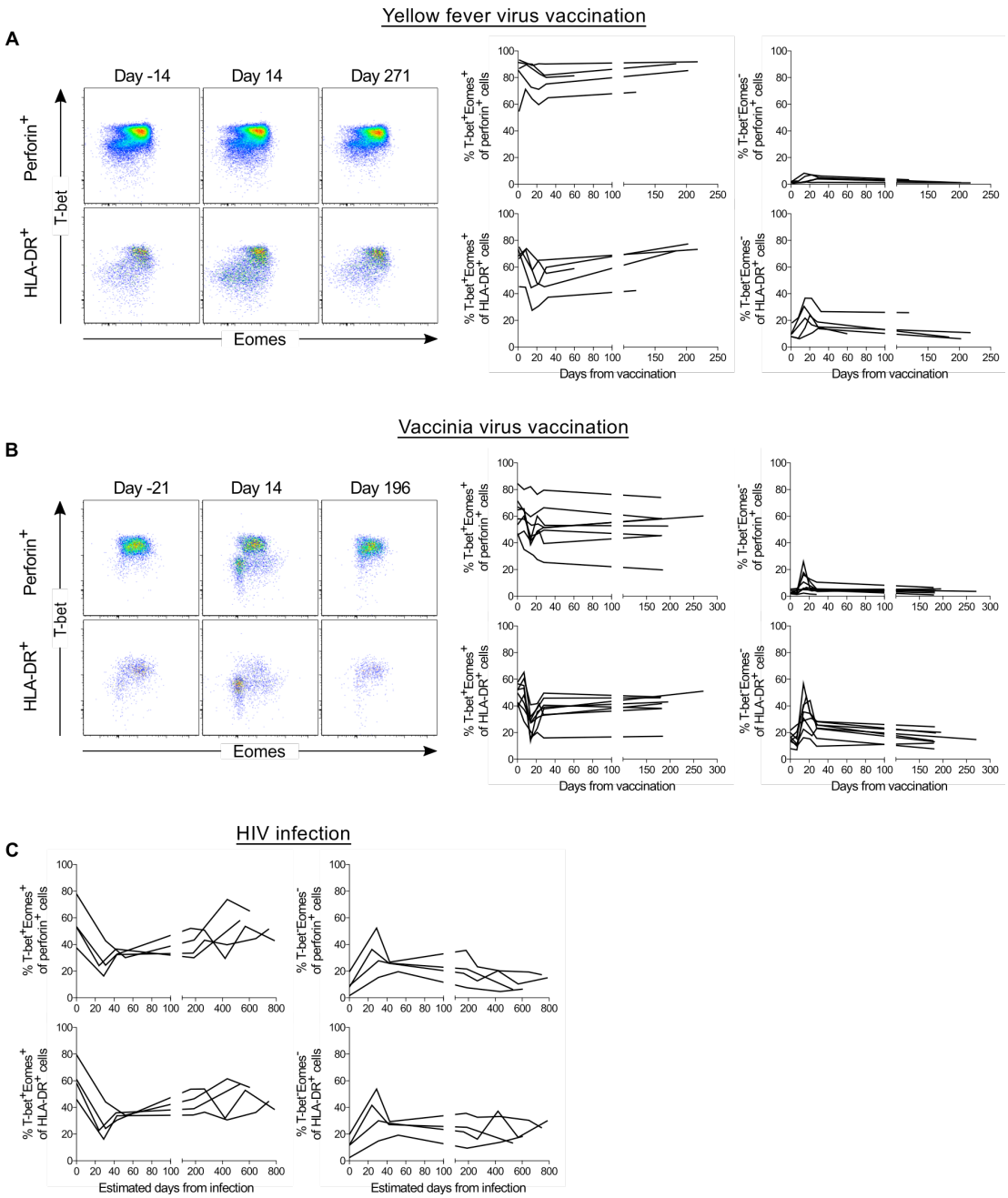

Figure 10. Loss of T-bet and Eomes expression by perforin$^+$ or HLA-DR$^+$ cells over the course of infection with yellow fever, vaccinia, or HIV. (**A**) T-bet and Eomes expression over the course of yellow fever vaccination for perforin$^+$ (top) or HLA-DR$^+$ (bottom) memory CD8$^+$ T cells. Representative flow cytometric plots for one donor; T-bet$^+$Eomes$^+$ and T-bet$^-$Eomes$^-$ subsets shown for all five donors. (**B**) T-bet and Eomes expression over the course of vaccinia vaccination for perforin$^+$ (top) or HLA-DR$^+$ (bottom) memory CD8$^+$ T cells. Representative flow cytometric plots for one donor; T-bet$^+$Eomes$^+$ and T-bet$^-$Eomes$^-$ subsets shown for all eight donors. (**C**) T-bet$^+$Eomes$^+$ and T-bet$^-$Eomes$^-$ subsets for perforin$^+$ (top) or HLA-DR$^+$ (bottom) memory CD8$^+$ T cells from four RV217 donors. All data represent direct *ex vivo* assessment with no *in vitro* stimulation.

HIV-specific cells retain T-bet and Eomes expression, but maintenance of cytotoxic potential over time is associated with higher per-cell T-bet levels

To determine if the transient loss of T-bet and Eomes within the bulk activated $CD8^+$ T cell memory pool during acute HIV infection extended to HIV-specific $CD8^+$ T cells, we assessed expression of these transcription factors in Gag-specific $CD8^+$ T cells. In marked contrast to the highly activated bulk $CD8^+$ T cell effector population during acute HIV infection, HIV-specific $CD8^+$ T cells expressed T-bet and/or Eomes at the earliest detectable time point and throughout the course of infection (**Fig. 11A-11C**). This indicates that despite their phenotypic similarities total and HIV-specific $CD8^+$ T cells may be primed quite differently during acute infection and raises the possibility that the majority of expanded effector $CD8^+$ T cells in early HIV infection may not be specific for HIV.

We next examined whether loss of perforin expression was related to changes in the level of T-bet expression during early HIV infection. Interestingly, the distribution of T-bet within Gag-specific $CD8^+$ T cells changed over time from acute to chronic infection (**Fig. 11D**). In the acute phase, responding cells were equally distributed between T-$bet^{Hi}Eomes^+$ and T-$bet^{Lo}Eomes^+$ expression patterns, which during the chronic phase began to be dominated by T-$bet^{Lo}Eomes^+$ cells (**Fig. 11D**). Furthermore, T-$bet^{Hi}Eomes^+$ HIV-specific $CD8^+$ T cells continued to express perforin as infection progressed, whereas T-$bet^{Lo}Eomes^+$ cells gradually lost perforin expression over time (**Fig. 11E and 11F**).

Finally, in contrast to the recent findings by Ndholuvu, et al. (Ndhlovu et al., 2015), we did not find the magnitude, proportion $perforin^+$, or any T-bet- or Eomes-

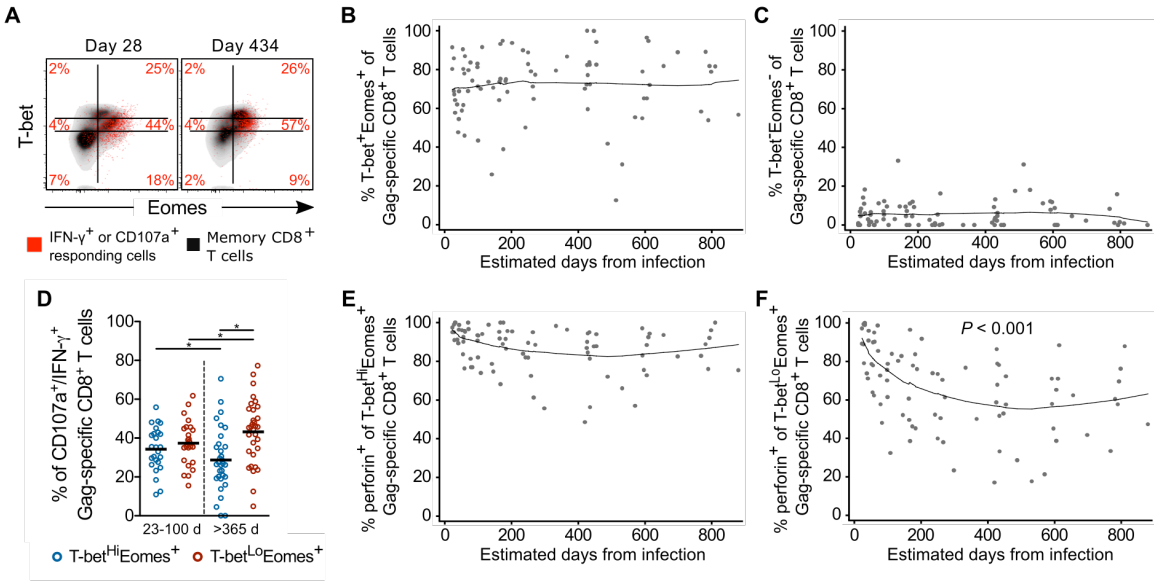

Figure 11. T-bet and Eomes expression by responding HIV-specific CD8$^+$ T cells over time. (**A**) Gag-specific CD8$^+$ T cells (red) overlaid on perforin$^+$ CD8$^+$ T cells (black) from the acute (day 28) and chronic (day 434) infection time points of one donor. Percentages of responding Gag-specific cells within each T-bet and Eomes subset are provided. Frequencies of T-bet$^+$Eomes$^+$ (**B**) and T-bet$^-$Eomes$^-$ (**C**) responding Gag-specific CD8$^+$ T cells over time. (**D**) Frequencies of T-bet^{Hi}Eomes$^+$ (blue circles) and T-bet^{Lo}Eomes$^+$ (red circles) responding Gag-specific CD8$^+$ T cells at acute (23-100 days) and chronic (>365 days) time points. Proportion of T-bet^{Hi}Eomes$^+$ (**E**) and T-bet^{Lo}Eomes$^+$ (**F**) responding Gag-specific CD8$^+$ T cell subsets that express perforin over time. * denotes a *P* value < 0.05. Statistics based on a GEE population-averaged model with Holm adjusted *P* value or random-effects tobit regression. Bars represent approximations of the means generated by the models. Lowess smoothers were used to represent the mean over time for longitudinal data.

expressing subset of responding HIV-1 Gag-specific $CD8^+$ T cells to be predictive of peak or set point viral load (data not shown). Despite this, our data suggest that in the earliest phase of infection, HIV-specific $CD8^+$ T cells have both the transcriptional and functional properties associated with long-term control of HIV replication (Hersperger et al., 2011; Hersperger et al., 2010), and that the inability to durably maintain high-level T-bet expression contributes to a qualitatively inferior response as infection progresses.

Discussion

Mechanisms underlying the inability of $CD8^+$ T cells to fully control HIV replication have remained unclear. Failure of antiviral immunity has been attributed in part to qualitative defects in total and HIV-specific $CD8^+$ T cells (Appay et al., 2000; Betts et al., 2006; Hersperger et al., 2010; Jansen et al., 2004; Trimble and Lieberman, 1998). However, the dysfunction observed within the $CD8^+$ T cell pool has largely been defined in the context of chronic infection when the success or failure of the presumed response has already been determined. The question of whether $CD8^+$ T cells in progressive infection were intrinsically less functional from the outset or if dysfunction arose over time has remained unanswered. To address this issue, we assessed the longitudinal $CD8^+$ T cell responses of a diverse cohort of individuals experiencing acute/early HIV infection. We show that acute HIV infection elicits a robust cytotoxic $CD8^+$ T cell response characterized by cells that express the cytolytic effector molecule perforin and the effector-associated transcription factors T-bet and Eomes. Importantly, the quality of the response quickly waned following the resolution of acute viremia, with a significant decrease in perforin expression by HIV-specific $CD8^+$ T cells that was at

least partially accounted for by a shift from T-bet^{Hi}Eomes^{+} cells to T-bet^{Lo}Eomes^{+} cells. The attenuation of the cytolytic response may help explain the failure of CD8^{+} T cells to control HIV replication in the long-term.

It is well documented that CD8^{+} T cell responses are elicited early in HIV infection and are associated with control of viral replication (Borrow et al., 1994; Koup et al., 1994; McMichael et al., 2010; Ndhlovu et al., 2015). Some of the strongest evidence of the CD8^{+} T cell-mediated immunologic pressure exerted during this period is the rapid emergence of viral escape mutations within known CD8^{+} T cell epitopes (Borrow et al., 1997; Fischer et al., 2010; Goonetilleke et al., 2009a). We found that HIV-specific cells had high cytotoxic potential at the earliest time points following HIV infection, but rapidly lost this function as disease progressed. This suggests a mechanism through which CD8^{+} T cells may exert a strong direct selective pressure on the virus resulting in the rapid selection of escape variants early in infection that ultimately have a reduced capacity to stimulate cytolytic CD8^{+} T cell responses (Fischer et al., 2010; Goonetilleke et al., 2009a; Liu et al., 2013; Sunshine et al., 2015). It should be noted that whereas perforin expression was lost over time almost all HIV-specific responding cells continued to produce MIP-1α. Thus, while cytotoxic CD8^{+} T cells play an important role in the resolution of acute viremia, as they lose their ability to express perforin they may be able to keep the virus partially in check through a combination of the remaining cytotoxic response and non-cytotoxic inhibitory effects exerted via the continued expression of β-chemokines or other non-cytolytic mechanisms (Levy, 2003). This would be consistent with models suggesting CD8+ T cell cytotoxic mechanisms do not account for the entirety of CD8^{+} T cell-mediated viral suppression during chronic progressive SIV

infection (Klatt et al., 2010; Wong et al., 2010). It remains unclear if maintenance of perforin expression following acute infection would further enhance the level of control over viral replication $CD8^+$ T cells provide as we would predict it should based on studies of $CD8^+$ T cell responses in the chronic phase of infection (Hersperger et al., 2010; Migueles et al., 2008; Saez-Cirion et al., 2007). Unfortunately, we were unable to find any direct associations between HIV-1 Gag-specific perforin, T-bet, or Eomes expression and the level of plasma viremia or $CD4^+$ T cell numbers.

T-bet and Eomes are important regulators of effector $CD8^+$ T cell differentiation and function for both mice and humans (Blom et al., 2015; Cruz-Guilloty et al., 2009; Hersperger et al., 2011; Joshi et al., 2007; Makedonas et al., 2010; McLane et al., 2013; Paley et al., 2012; Pearce et al., 2003; Sullivan et al., 2003; van Aalderen et al., 2015). Expression patterns of these transcription factors have been described for $CD8^+$ T cells in the context of various human viral infections, including CMV, EBV, HBV, HCV, HIV, and TBEV (Blom et al., 2015; Buggert et al., 2014; Greenough et al., 2015; Hersperger et al., 2011; Hertoghs et al., 2010; Kurktschiev et al., 2014; Makedonas et al., 2010; Paley et al., 2012; Popescu et al., 2014). These studies demonstrated a high degree of variability in the relative levels of T-bet and Eomes expressed by virus-specific $CD8^+$ T cells depending on time from infection, whether the infection was controlled, and tissue localization. CMV-specific cells express T-bet and Eomes during both acute and chronic phases of infection, but control of viral replication in the acute phase is associated with a higher ratio of T-bet^+ versus $Eomes^+$ cells (Hertoghs et al., 2010; Popescu et al., 2014). EBV- and TBEV-specific cells also express T-bet and Eomes during the earliest phase of their respective infections, but EBV-specific cells lose expression of both during

convalescence whereas TBEV-specific cells retain T-bet expression and show a gradual reduction in Eomes (Blom et al., 2015; Greenough et al., 2015). HCV-specific cells are T-bet^{+} in acute/resolving HCV infection and T-bet^{-}Eomes^{-} during acute/non-resolving infection. Post-acute phase, HCV-specific cells in the peripheral blood are T-bet^{-}Eomes^{-} for both resolved and non-resolved HCV infection, but T-bet^{+} within the livers of subjects with resolved infection and Eomes^{+} in livers of chronically infected subjects (Kurktschiev et al., 2014; Paley et al., 2012). Together, these results suggest expression of T-bet during the acute phase is a critical determinant of viral infection outcome. The differential outcomes associated with Eomes were also reflective of the relative expression level of T-bet, suggesting Eomes may not be as important for the resolution of acute viremia. Rather, Eomes expression may determine whether antigen-specific cells are fated to form a stable memory pool or become exhausted subsequent to the acute phase, dependent on whether or not the infection is ultimately cleared (Doering et al., 2012; Paley et al., 2012).

Similar associations between T-bet, Eomes, and outcome have been demonstrated in chronic HIV infection. In this context, a high level of T-bet expression was associated with greater overall functionality of HIV-specific CD8^{+} T cells, including cytotoxic potential, and relative control of viral replication, whereas low T-bet levels and continued Eomes expression has been associated with lower overall functionality and persistent viremia (Buggert et al., 2014; Hersperger et al., 2011). Our data show that HIV-specific cells have high cytotoxic potential during acute infection, but lose the ability to express or rapidly upregulate perforin in chronic infection. This loss of cytotoxic potential over time can at least partially be explained by a change in the relative expression levels of T-

bet and Eomes: HIV-specific cells were equally T-bet^{Hi}Eomes^{+} and T-bet^{Lo}Eomes^{+} during acute infection and both subsets efficiently upregulated perforin initially but the proportion of T-bet^{Lo}Eomes^{+} cells increased significantly as infection progressed and cells with this phenotype had an inferior capacity to express perforin compared to T-bet^{Hi}Eomes^{+} cells. The expression of perforin by either phenotype during acute infection may be reflective of the high degree of inflammation and activation during this phase, a differential role for Eomes at different stages of infection, and/or the result of additional transcription factors not assessed here. Whatever the case may be, T-bet^{Hi}Eomes^{+} HIV-specific CD8^{+} T cells retain the ability to upregulate perforin following resolution of acute viremia and this subset declines during chronic progressive infection.

Recent data from Ndhlovu *et al.* suggests HIV infection elicits a massive antigen-specific CD8^{+} T cell response with limited bystander activation (Ndhlovu et al., 2015). Similar observations have been reported after vaccination with vaccinia and yellow fever virus (Miller et al., 2008). The similarities in differentiation state, activation, and immediate cytotoxic potential between total peripheral memory and Gag-specific cells reported here support the idea of a robust and specific response to HIV infection. However, we found a significant discrepancy between transcriptional control of HIV-specific CD8^{+} T cells versus the bulk activated perforin^{+} memory CD8^{+} T cell population. The degree to which these differences reflect a true lack of specificity, dysfunction on the part of the bulk activated cells, an inability to identify an appropriate functional marker, or an attempt by the host to mitigate immune-mediated pathology remains unclear. It is likely there area many more circulating HIV-specific CD8^{+} T cells than indicated by our findings using *in vitro* stimulation with only two HIV-1 proteins

and a limited number of functional parameters to identify responding cells. However, it should be noted that $CD8^+$ T cell bystander activation has been reported during acute HIV and EBV infection in humans and it is possible at least a subset of $CD8^+$ T cells are activated non-specifically in our cohort (Doisne et al., 2004; Odumade et al., 2012). T cell receptor stimulation is required for upregulation of T-bet (Szabo et al., 2000), but a large proportion of bulk activated $perforin^+$ cells during acute HIV infection appear to express neither T-bet nor Eomes whereas all Gag-specific cells expressed one or the other. In addition, perforin can be upregulated in the absence of direct antigenic stimulation via exposure to IFN-α (Kohlmeier et al., 2010), levels of which are highly elevated during acute HIV infection (Stacey et al., 2009). Thus, the difference in T-bet and Eomes expression we observed between bulk $perforin^+$ and responding HIV-specific $CD8^+$ T cells raises the possibility that a significant number of bystander-activated cells are being induced in response to HIV infection. Alternatively, given the association between activation and the size of the $T\text{-}bet^-Eomes^-$ pool across infections with vaccinia, yellow fever, and HIV, the absence of T-bet and Eomes expression in the bulk $perforin^+$ $CD8^+$ T cell pool may be a characteristic of the contraction phase that typically follows the initial $CD8^+$ T cell response. This would be consistent with the pro-apoptotic phenotype of the majority of cells following peak HIV viremia and the timing of our samples (Ndhlovu et al., 2015). Whether HIV-specific or bystander, the lack of T-bet and Eomes expression by these cells suggests they would be unable to sustain perforin expression upon encountering infected target cells. This may in part explain the inability of bulk peripheral $CD8^+$ T cells from acutely HIV infected individuals to efficiently inhibit viral replication *in vitro* and further suggests they would not make a meaningful

contribution to long-term control of viral replication *in vivo* (Eller et al., 2016; Lecuroux et al., 2013).

These data show how the peripheral $CD8^+$ T cell response to HIV evolves over the course of progressive infection. HIV-specific $CD8^+$ T cells are able to upregulate perforin and T-bet initially but begin to lose this capacity soon after peak viremia, demonstrating for the first time that there is not an initial intrinsic inability of HIV-specific $CD8^+$ T cells to upregulate these molecules. It remains unclear how or if these responses differ from those of $CD8^+$ T cells from subjects who go on to spontaneously control viral replication to very low levels in the chronic phase. While we did find frequencies of T-bet^+ and T-bet^-Eomes^- total memory $CD8^+$ T cells at set point time points were inversely or directly associated with set point viral load, respectively, we did not find any associations between viral load and the size of the total peripheral $perforin^+$ pool or the magnitude or cytotoxic potential of HIV-1 Gag-specific cells at any time point. Nor did we find any subset of total memory or Gag-specific cells to be predictive of set point viral load for this group of subjects, possibly due to the limited number of very early time points and relatively narrow range of viral loads at set point. However, the fact that the initial phenotype of HIV-specific cells is similar to that associated with control during the chronic phase of infection suggests induction and maintenance of cells capable of upregulating high levels of T-bet and perforin could lead to subsequent control. Eliciting HIV-specific cells with these characteristics might serve as an important target for vaccination or therapeutic modalities seeking to fully control early viral replication or eradicate the chronic viral reservoir.

CHAPTER 3

$CD8^+$ T CELL PERFORIN EXPRESSION INDEPENDENT OF T-BET OR EOMES DURING CHRONIC PROGRESSIVE HIV INFECTION

Summary

Recent data suggest that $CD8^+$ T cell effector activity is an important component to the control of HIV replication in elite controllers (EC). Two critical regulators of $CD8^+$ T cell effector differentiation and function are the T-box transcription factors T-bet and Eomes. Here, we assessed T-bet and Eomes expression, together with the cytolytic protein perforin in $CD8^+$ T cells from EC, chronic progressors (CP), and antiretroviral therapy-suppressed individuals (HAART). We found increased frequencies of perforin-expressing cells in both the total memory and HIV-specific $CD8^+$ T cell pools from CP when compared to the other groups. We did not observer significant differences in the expression patterns of T-bet or Eomes for $CD8^+$ T cells from any of the groups. However, whereas perforin expression was strongly associated with T-bet and Eomes in EC, CP $CD8^+$ T cells expressed perforin even in the absence of either transcription factor. Notably, CP in whom the associations were relatively intact demonstrated greater *in vivo* control of viral replication. Collectively, these results suggest that maintenance of the relationships between perforin, T-bet, and Eomes may be more important for control than the absolute expression of any one factor on its own.

Introduction

The majority of HIV-infected individuals experience high viral loads and a progressive loss of $CD4^+$ T cells that in the absence of antiretroviral therapy (ART) results in severe immunodeficiency and death due to AIDS-related complications. While access to ART is increasing globally it remains unavailable to many (UNAIDS, 2016), is associated with increased non-AIDS-related morbidity and mortality (Choi et al., 2009; Phillips et al., 2008), and treatment interruption results in rapid rebound of viral replication (Davey et al., 1999; Rothenberger et al., 2015). As such, a functional cure that can provide long-term control of viral replication in the absence of therapy remains an area of intense research interest (Deeks et al., 2016). A rare subset of HIV-infected individuals known as "elite controllers" (ECs) spontaneously control viral replication and may serve as a natural model for cure strategies (Lambotte et al., 2005; Pereyra et al., 2008). Understanding the mechanisms by which ECs control HIV may provide insight for the development of vaccine modalities and novel immunotherapeutics.

Several lines of evidence suggest that $CD8^+$ T cells play an important role in controlling HIV replication. These include the temporal association between the resolution of acute viremia and expansion of HIV-specific $CD8^+$ T cells (Borrow et al., 1994; Koup et al., 1994), the emergence of viral escape mutations in $CD8^+$ T cell epitopes (Borrow et al., 1997; Goonetilleke et al., 2009b; Goulder et al., 1997; Liu et al., 2013; Price et al., 1997), and strong correlations between certain HLA class I alleles and disease progression (Carrington and O'Brien, 2003; Migueles et al., 2000; Scherer et al., 2004). Notably, studies comparing $CD8^+$ T cells from ECs and individuals with chronic progressive infection (CPs) have shown that the differential ability to control viral

replication cannot be explained by differences in frequencies of HIV-specific cells or in their capacities to recognize $CD4^+$ T cells infected with autologous virus (Draenert et al., 2004; Migueles et al., 2003; Migueles et al., 2004). On the other hand, several differences in the qualitative features of HIV-specific $CD8^+$ T cell responses have been associated with control. Cells from ECs have a greater capacity to proliferate (Migueles et al., 2002; Migueles et al., 2009; Zimmerli et al., 2005), are more polyfunctional (Almeida et al., 2007; Betts et al., 2006), and have greater cytotoxic capacity (Appay et al., 2000; Chen et al., 2009; Hersperger et al., 2010; Migueles et al., 2009; Saez-Cirion et al., 2007). The primary mechanism by which $CD8^+$ T cells kill virally infected cells is via the exocytosis of granules containing the cytolytic proteins perforin and granzyme B (Barry and Bleackley, 2002; Podack, 1989). Control of HIV replication has been linked to a greater capacity of HIV-specific $CD8^+$ T cells to upregulate these effector molecules during *in vitro* culture or following brief stimulation directly *ex vivo* (Hersperger et al., 2010; Migueles et al., 2008).

Murine models identified the T-box transcription factors T-bet and Eomesodermin (Eomes) as key regulators of effector $CD8^+$ T cell differentiation and function (Cruz-Guilloty et al., 2009; Kaech and Cui, 2012; Pearce et al., 2003; Sullivan et al., 2003). T-bet drives terminal differentiation of $CD8^+$ T cells and is associated with the expression of IFN-γ, granzyme B, and perforin (Buggert et al., 2014; Hersperger et al., 2011; Intlekofer et al., 2007; Jenner et al., 2009; Joshi et al., 2007; Makedonas et al., 2010). Eomes is associated with granzyme B and perforin but also with the expression of proteins involved in the maintenance of memory $CD8^+$ T cells (Banerjee et al., 2010; Cruz-Guilloty et al., 2009; Joshi et al., 2007; Pearce et al., 2003). Thus although there is a

level of redundancy with respects to their roles in driving some effector molecules, T-bet and Eomes have reciprocal roles in regulating effector and memory differentiation pathways. In the context of chronic HIV infection, perforin expression was shown to correlate directly with T-bet content, and HIV-specific $CD8^+$ T cells from ECs preferentially maintained higher levels of T-bet compared to CPs (Buggert et al., 2014; Hersperger et al., 2011). Eomes was recently reported to have an inverse association with perforin expression in CPs but their relationship has not been examined in controllers (Buggert et al., 2014). Thus it remains unclear if Eomes expression either alone or in combination with T-bet further differentiates the effector $CD8^+$ T cell response during controlled and uncontrolled HIV infection.

To address this issue, we examined the relationship between effector $CD8^+$ T cell responses and T-bet and Eomes expression in a cross-sectional cohort of individuals who differentially control HIV replication naturally or fully suppress viremia by means of antiretroviral therapy. We found T-bet and Eomes expression patterns to be largely similar between controllers and noncontrollers for both total resting memory and HIV-specific $CD8^+$ T cells. However, whereas there were strong direct associations between $CD8^+$ T cell perforin expression and both T-bet and Eomes in controllers, these relationships were dysregulated during chronic progressive infection. Importantly, amongst individuals with progressive infection, maintenance of perforin$^+$ $CD8^+$ T cells with T-bet and Eomes expression patterns similar to those found in ECs correlated with lower viral loads.

Material and Methods

Study participants: We examined HIV-specific $CD8^+$ T cells responses from a cross-sectional cohort of 20 elite controllers, 33 chronic progressors, and 20 HAART-suppressed individuals. The majority of peripheral blood mononuclear cell (PBMC) samples were obtained through clinics associated with Harvard University. Thirteen CP PBMC samples were obtained from the Center for Research on Infectious Disease (CIENI), National Institute of Respiratory Disease, Mexico DF, Mexico. All PBMC samples were obtained in compliance with the guidelines established by the institutional review board for each site. EC were defined as therapy-naïve individuals who had consistent plasma HIV RNA levels below the limit of detection (< 50 copies/ml) for a minimum of three measurements during at least a 12-month period. CP were defined as untreated individuals with plasma HIV RNA levels consistently above 10,000 copies/ml. HAART-suppressed individuals maintained plasma HIV RNA levels below the limit of detection (< 50 copies/ml) for a minimum of two years. $CD4^+$ T cells counts were not considered as inclusion criteria for any of the groups.

Peptides: Potential T cell epitope (PTE) peptides corresponding to the HIV-1 Gag and Nef proteins were obtained from the NIH AIDS Reagent Program (NIH, Bethesda, Maryland). PTE peptides are 15 amino acids in length and contain naturally occurring 9 amino acid sequences that are potential T cell determinants embedded in the sequences of circulating HIV-1 strains worldwide. Human cytomegalovirus (CMV) IE1 and pp65 proteins were synthesized by New England Peptide (Gardner, MA). Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis/Missouri, USA),

combined into pools at 400 μg/ml, and stored at -20°C prior to use.

PBMC stimulation: Cryopreserved PBMCs were thawed and rested overnight at $2x10^6$ cells/ml in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cell viability was checked both immediately after thawing and after overnight rest by trypan blue exclusion. Costimulatory antibodies (anti-CD28 and anti-CD49d, 1 μg/mL each; BD Biosciences) and pre-titrated fluorophore conjugated anti-CD107a were included at the start of all stimulations. PBMCs were incubated for 1 hour at 37°C and 5% CO_2 prior to the addition of monensin (1 μg/mL; BD Biosciences) and brefeldin A (10 μg/mL; Sigma-Aldrich) followed by an additional 5 hour incubation at 37°C and 5% CO_2. For peptide stimulations, peptides from the Gag, Nef, or CMV pools were added to separate tubes of cells such that each individual peptide was at a final concentration of 1 μg/ml. As a negative control, DMSO was added to the cells at an equivalent concentration to the one used for peptide stimulation.

Antibody reagents: Antibodies for surface staining included CCR7 APC-eFluor780 (clone 3D12; eBioscience), CD4 PE-Cy5.5 (clone S3.5; Invitrogen), CD8 BV711 (clone RPA-T8; Biolegend), CD14 PE-Cy5 (clone 61D3; Abcam), CD16 PE-Cy5 (clone 3G8; Biolegend), CD19 PE-Cy5 (clone HIB19; Biolegend), CD27 BV785 (clone O323; Biolegend), CD45RO ECD (clone UCHL1; Beckman Coulter), and CD107a PE-Cy7 (clone H4A3; Biolegend). Antibodies for intracellular staining included: CD3 BV650

(clone UCHT1; Biolegend), Eomes eFluor 660 (WD1928; eBioscience), IFN-γ Alexa 700 (clone B27; Invitrogen), MIP-1α FITC (clone 93342; R&D Systems), Perforin BV421 (clone B-D48, Biolegend), and T-bet PE (clone 4B10; eBioscience).

Flow cytometric analysis: At the end of the stimulations, cells were washed once with PBS prior to be being stained for CCR7 expression for 15 min at 37°C in the dark. Cells were then stained for viability with aqua amine-reactive viability dye (Invitrogen) for 10 min at room temperature in the dark followed by addition of a cocktail of antibodies to stain for surface markers for an additional 20 min. The cells were washed with PBS containing 0.1% sodium azide and 1% BSA, fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), and stained with a cocktail of antibodies against intracellular markers for 1 h at room temperature in the dark. The cells were washed once with Perm Wash buffer (BD Biosciences) and fixed with PBS containing 1% paraformaldehyde. Fixed cells were stored at 4°C in the dark until acquisition. Antibody capture beads (BD Biosciences) were used to prepare individual compensation controls for each antibody used in the experiment. ArC Amine Reactive beads (ThermoFisher Scientific) were used to generate a singly stained compensation control for the aqua amine-reactive viability dye.

A minimum of 250,000 total events were acquired for each stimulation conditionusing a modified LSRII (BD Immunocytometry Systems). Data analysis was performed using FlowJo (TreeStar) software. Reported antigen-specific data have been corrected for background based on the negative (no peptide) control, and only responses with a total frequency twice the negative control and above 0.01% of total memory $CD8^+$

T cells (after background subtraction) were considered to be positive responses. By analyzing the data in this way, we examined cytolytic protein production resulting from antigen-specific stimulation and ensured that its expression was considered only within responding CD8 T cells expressing at least one other functional parameter. IFN-γ, CD107a, and MIP-1α were used to identify antigen-specific $CD8^+$ T cells and figures depicting antigen-specific data were derived from analysis of cells expressing any one of these three markers.

Statistical analysis: All statistical analyses were performed and graphs were generated using GraphPad Prism (version 5.0a). Nonparametric tests were used for all comparisons between study groups (Mann-Whitney test for two groups; Kruskal-Wallis test followed by Dunns post test for multiple comparisons when comparing three or more groups). Correlations were determined using Spearman's rank correlation test (non-parametric; two-tailed). Differences were considered significant if the *P* value was below 0.05. For all figures, * denotes a *P* value < 0.05, ** denotes a *P* value < 0.01, and *** denotes a *P* value < 0.001.

Results

A greater proportion of total memory peripheral $CD8^+$ T cells from CP expressed perforin directly *ex vivo* compared to EC or HAART

Initially we assessed the differentiation state of total resting peripheral $CD8^+$ T cells from 20 elite controllers (EC), 33 chronic progressors (CP), and 20 individuals suppressing HIV replication through antiretroviral therapy [HAART] (**Table 2**). Using multiparameter flow cytometry we were able to identify six populations within the circulating $CD8^+$ T cell pool using combinations of the markers CCR7, CD27, and CD45RO: naïve ($CCR7^+CD27^+CD45RO^-$), central memory (CM; $CCR7^+CD27^+CD45RO^+$), transitional memory (TM; $CCR7^-CD27^+CD45RO^+$), intermediate memory (Int; $CCR7^-CD27^+CD45RO^-$), effector memory (EM; $CCR7^-CD27^-CD45RO^+$), and effector (Eff; $CCR7^-CD27^-CD45RO^-$) cells (**Fig. 12A**). There were no significant differences in the proportion of cells with a naïve phenotype between the three groups but there were differences in the distributions of memory subsets (**Fig. 12B**). Notably, progressors had a greater proportion of cells with an effector memory phenotype compared to controllers and ART-suppressed individuals, whereas progressors and controllers had similar proportions of cells with the more fully differentiated $CCR7^-CD27^-CD45RO^-$ phenotype. These data suggested that $CD8^+$ T cells from progressors were in general more differentiated than cells from controllers.

We next examined the perforin content of the total resting memory $CD8^+$ T cell pool for each group. We found a greater frequency of $perforin^+$ $CD8^+$ T cells in progressors (**Fig. 12 C and D**), in agreement with the larger proportion of cells with a more differentiated status in these individuals and reported perforin expression patterns

Table 2. Clinical parameters of the HIV chronic infection study cohort

	Elite Controllers	Chronic Progressors	HAART-suppressed
Number of subjects	20	33	20
Plasma HIV RNA, **median** (*IQR*), copies/ml	Not detectable	**45300** (*20526-132927*)	Not detectable
CD4+ T cell count, **median** (*IQR*), cells/mm^3	**732** (*536-1136*)	**480** (*385-636*)	**941** (*735-1236*)
Infection duration, **median** (*IQR*), years	**18** (*11-23*)	N.D.	**16** (*12-21*)
Duration of HAART, **median** (*IQR*), years	N/A	N/A	**7** (*3-12*)

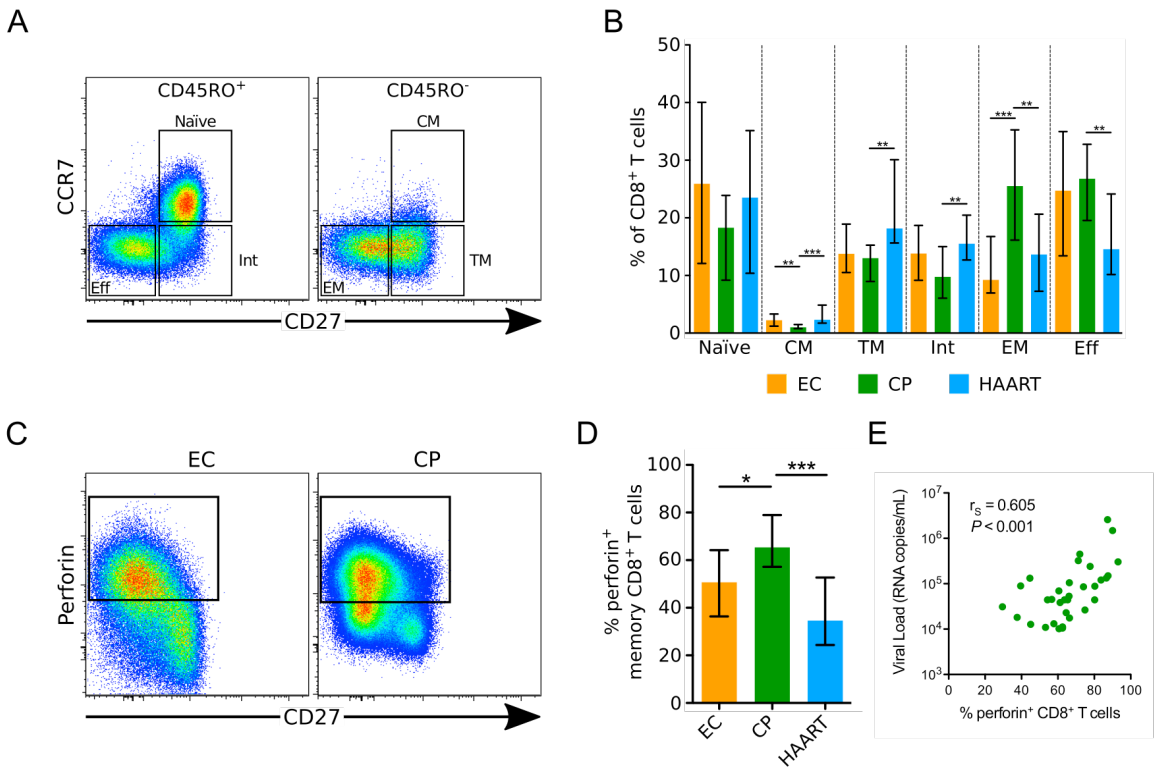

Figure 12. Memory distributions and proportions of perforin$^+$ peripheral CD8$^+$ T cells directly *ex vivo*. (**A**) Representative flow cytometric plots of CD45RO, CCR7, and CD27 profiles for total CD8$^+$ T cells and the gating strategy to identify naïve (CCR7$^+$CD27$^+$CD45RO$^-$), central memory (CM; CCR7$^+$CD27$^+$CD45RO$^+$), transitional memory (TM; CCR7$^-$CD27$^+$CD45RO$^+$), intermediate memory (Int; CCR7$^-$CD27$^+$CD45RO$^-$), effector memory (EM; CCR7$^-$CD27$^-$CD45RO$^+$), and effector (Eff; CCR7$^-$CD27$^-$CD45RO$^-$) cells. (**B**) Memory subset distributions for EC (yellow), CP (green), and HAART (blue). (**C**) Representative flow cytometric plots showing perforin expression by memory CD8$^+$ T cells for one EC and one CP. (**D**) Proportion of memory CD8$^+$ T cells that express perforin for all EC, CP, and HAART. (**E**) Viral load plotted against the proportion of memory CD8$^+$ T cells that express perforin for CP. * denotes a P value < 0.05, ** denotes a P value < 0.01, and *** denotes a P values < 0.001. Statistics based on a Kruskal-Wallis test followed by Dunns post test for multiple comparisons. Correlation was determined using Spearman's rank correlation test (non-parametric; two-tailed).

(Chattopadhyay et al., 2009; Takata and Takiguchi, 2006). Upon further investigation of the progressors group, we also found a strong direct correlation between the frequency of perforin^{+} CD8^{+} T cells and plasma viral load (**Fig. 12 E**). Together these data suggested CD8^{+} T cells from CPs are potentially more cytolytic than cells from controllers and this may be driven in part by HIV replication.

Larger frequency of perforin^{+} CD8^{+} T cells in CP was not associated with enhanced expression of T-bet or Eomes

We and others recently reported that CD8^{+} T cell differentiation in humans is related to the expression levels of T-bet and Eomes within in a cell (Hersperger et al., 2011; McLane et al., 2013; van Aalderen et al., 2015). Cells with more T-bet have an effector memory or effector phenotype whereas cells with low levels of T-bet and more Eomes have a central memory or transitional memory phenotype. In addition, perforin expression has previously been shown to be directly associated with T-bet expression (Buggert et al., 2014; Hersperger et al., 2011; Makedonas et al., 2010). Given the more mature memory phenotype and greater proportion of perforin^{+} CD8^{+} T cells we observed in the progressors, we expected to find a greater frequency of T-bet^{Hi} CD8^{+} T cells in these individuals compared to controllers. However, upon examination we did not find any significant differences in any of the T-bet subsets between the groups (**Fig. 13 A and B**). When we assessed the relationship between perforin and T-bet expression we found strong direct correlations for controllers and HAART-suppressed individuals but not for progressors (**Fig. 13C**). CD8^{+} T cells from noncontrollers instead appeared to express more perforin than would have been predicted based on their T-bet content.

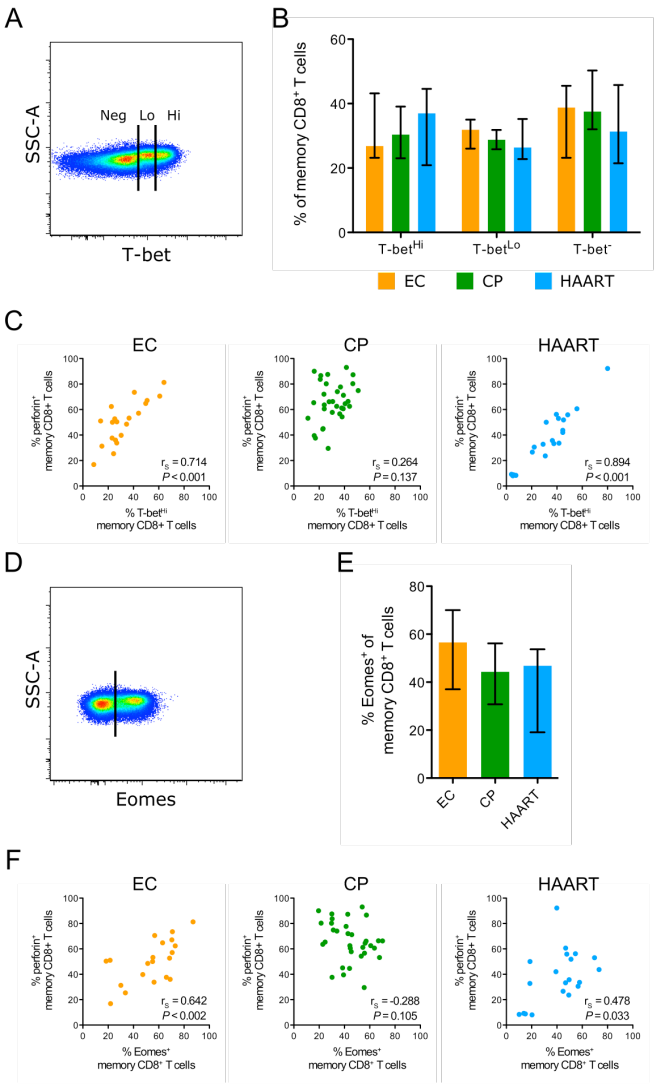

Figure 13. T-bet and Eomes expression by total peripheral memory CD8$^+$ T cells. (**A**) Representative flow cytometric plot of T-bet expression by CD3$^+$CD8$^+$ T cells from one EC showing three populations typically observed in human cells. (**B**) T-bet subset distributions for memory CD8$^+$ T cells from all EC (yellow), CP (green), and HAART (blue). (**C**) Proportion of perforin$^+$ memory CD8$^+$ T cells plotted against the proportion of T-bet^{Hi} memory CD8$^+$ T cells for EC, CP, and HAART. (**D**) Representative flow cytometric plot of Eomes expression by CD3$^+$CD8$^+$ T cells from one EC showing the bimodal expression pattern typically observed in human cells. (**E**) Proportion of Eomes$^+$ memory CD8$^+$ T cells from all EC, CP, and HAART. (**F**) Proportion of perforin$^+$ memory CD8$^+$ T cells plotted against the proportion of Eomes$^+$ memory CD8$^+$ T cells for EC, CP, and HAART. There were no statistically significant differences for proportions of T-bet subset or Eomes$^+$ cells between groups. Statistics based on a Kruskal-Wallis test followed by Dunns post test for multiple comparisons. Correlations were determined using Spearman's rank correlation test (non-parametric; two-tailed).

Despite a recent report that perforin and Eomes expression share an inverse relationship in $CD8^+$ T cells during chronic progressive HIV infection (Buggert et al., 2014), murine models indicate Eomes can play a role in driving the development of cytolytic $CD8^+$ T cells and Eomes expression was associated with $perforin^+$ $CD8^+$ T cell responses during the early phase of tick-borne encephalitis virus infection in humans (Blom et al., 2015; Cruz-Guilloty et al., 2009; Pearce et al., 2003). As such, it was possible Eomes was responsible for the increased perforin expression observed in our group of progressors. However, as with T-bet, we did not find any differences in the frequencies of $Eomes^+$ memory $CD8^+$ T cells between the groups (**Fig 13 D and E**). We did find positive correlations between perforin and Eomes expression for controllers and HAART-suppressed individuals but, also similar to T-bet, this relationship was lost in progressors (**Fig. 13F**).

We next sought to determine if coexpression patterns of T-bet and Eomes, rather than either transcription factor on its own, would differentiate the total memory $CD8^+$ T cell pools between each group. Only the proportions of cells with a $T\text{-}bet^{Hi}Eomes^-$ expression profile were significantly different between progressors and controllers, and this population represented less than 10% of the total memory $CD8^+$ T cell pool (**Fig. 14 A and B**). This difference alone could not explain the much larger frequency of $perforin^+$ cells found in progressors. Also, while perforin expression positively correlated with cells with a $T\text{-}bet^{Hi}Eomes^+$ phenotype and negatively correlated with $T\text{-}bet^-Eomes^-$ cells in both EC and HAART-suppressed, there were no such correlations for progressors (**Fig 14 C and D**).

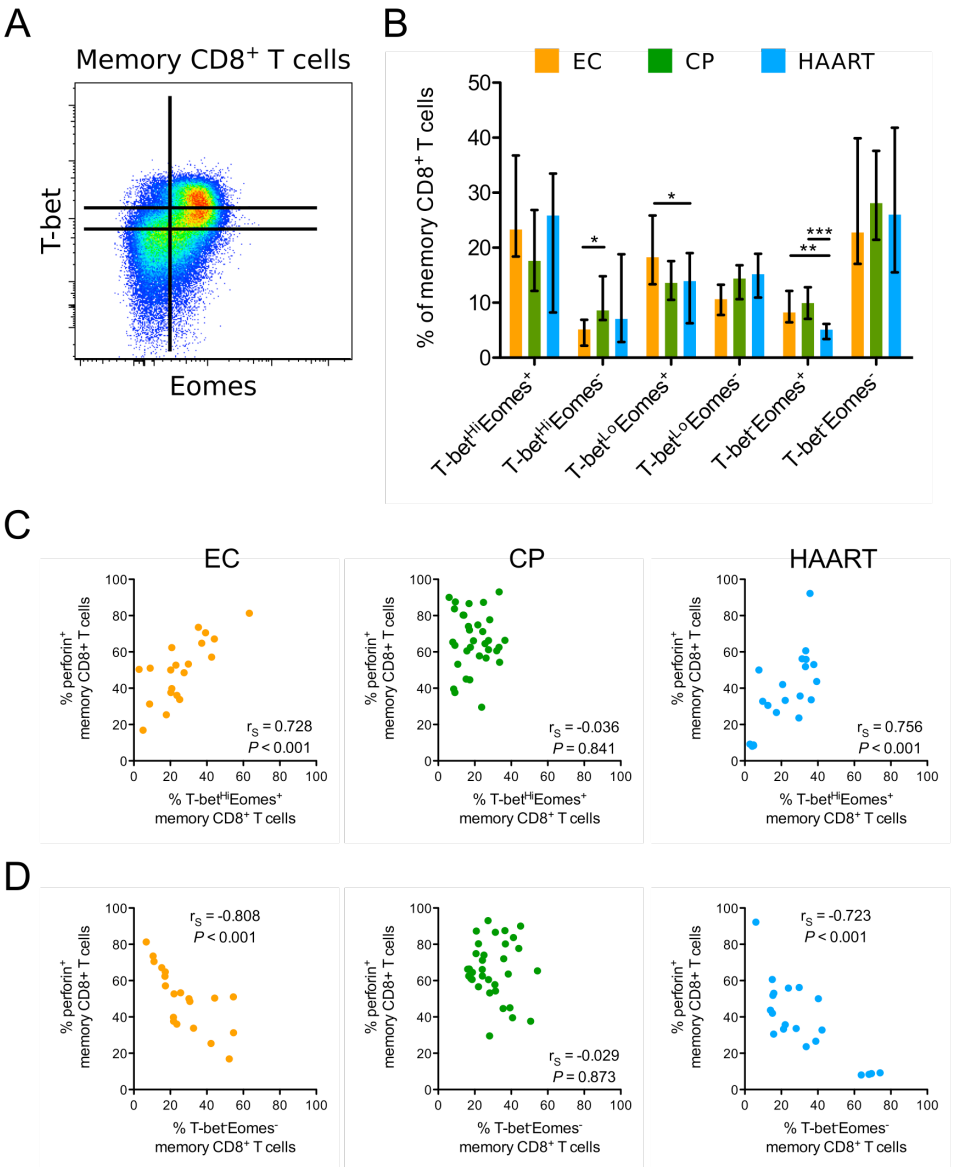

Figure 14. T-bet and Eomes coexpression patterns for total peripheral memory CD8$^+$ T cells. (**A**) Representative flow cytometric plot of T-bet and Eomes expression for total memory CD8$^+$ T cells from one EC. (**B**) T-bet and Eomes expression by total memory CD8$^+$ T cells for all EC (yellow), CP (green), and HAART (blue). (**C**) Proportion of perforin$^+$ memory CD8$^+$ T cells plotted against the proportion of T-bet^{Hi}Eomes$^+$ memory CD8$^+$ T cells for EC, CP, and HAART. (**D**) Proportion of perforin$^+$ memory CD8$^+$ T cells plotted against the proportion of T-bet$^-$Eomes$^-$ memory CD8$^+$ T cells for EC, CP, and HAART. * denotes a P value < 0.05, ** denotes a P value < 0.01, and *** denotes a P values < 0.001. Statistics based on a Kruskal-Wallis test followed by Dunns post test for multiple comparisons. Correlations were determined using Spearman's rank correlation test (non-parametric; two-tailed).

Frequencies of perforin^{+} CD8^{+} T cells are increased across multiple T-bet and Eomes subsets in CP compared to EC

The greater frequency of perforin^{+} CD8^{+} T cells and lack of association between perforin and T-bet or Eomes expression observed in progressors suggested that there was aberrant regulation of perforin expression within the peripheral CD8^{+} T cell pool during uncontrolled HIV infection. To examine the relationships between perforin, T-bet, and Eomes more directly, we assessed the relative frequencies of perforin^{+} CD8^{+} T cells for each T-bet and Eomes coexpression subset. For individuals with controlled viral replication, frequencies of perforin^{+} cells declined with lower levels of T-bet and/or absence of Eomes expression (**Fig. 15 A and B**). While progressors exhibited a similar pattern of decline, they also had greater frequencies of perforin^{+} cells across all subsets, including cells that expressed neither T-bet nor Eomes (**Fig. 15 A and B**).

We next examined the relationship between perforin^{+} CD8^{+} T cell T-bet and Eomes subsets and viral load. Amongst progressors, viral load inversely correlated with the frequencies of perforin^{+} cells that were T-bet^{Hi}, Eomes^{+}, and T-bet^{Hi}Eomes^{+} and directly correlated with T-bet^{-}Eomes^{-} perforin^{+} cells (**Fig. 15C**). These correlations were even stronger when controllers were included in the analysis (not shown). Together, these data indicate that maintenance of specific associations between the expression of perforin, T-bet, and Eomes within the total circulating memory CD8^{+} T cell pool is predictive of control of viral load, irrespective of progression status.

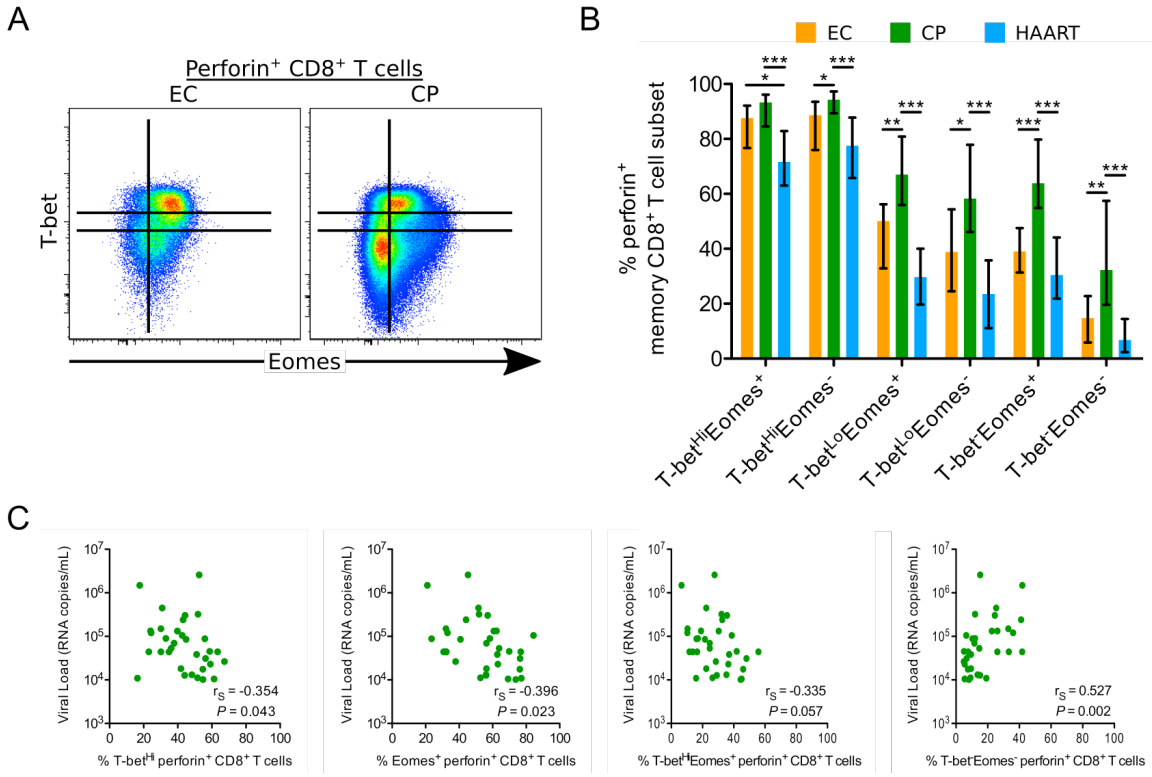

Figure 15. Perforin expression by total peripheral CD8$^+$ T cell T-bet and Eomes subsets. (**A**) Representative flow cytometric plot of T-bet and Eomes expression for total perforin$^+$ CD8$^+$ T cells from one EC. (**B**) T-bet and Eomes expression by total perforin$^+$ CD8$^+$ T cells for all EC (yellow), CP (green), and HAART (blue). (**C**) HIV viral load plotted against the proportion of perforin$^+$ CD8$^+$ T cells that were T-bet^{Hi}, Eomes$^+$, T-bet^{Hi}Eomes$^+$, or T-bet$^-$Eomes$^-$ for CP. * denotes a P value < 0.05, ** denotes a P value < 0.01, and *** denotes a P values < 0.001. Statistics based on a Kruskal-Wallis test followed by Dunns post test for multiple comparisons. Correlations were determined using Spearman's rank correlation test (non-parametric; two-tailed).

HIV-specific $CD8^+$ T cells from CP demonstrated a greater ability to express perforin than EC or HAART

We next sought to determine if differences identified between groups in the bulk memory $CD8^+$ T cell pool were consistent across HIV- and other virus-specific cells in general. We therefore stimulated PBMCs with peptide pools representing potential T cell epitopes from HIV Gag (p17 and p24) and Nef, or overlapping peptides from CMV representative of IE1 and pp65. Virus-specific responding cells were identified based on their ability to degranulate (express CD107a) or produce IFNγ or MIP-1α (**Fig. 16A**). The magnitudes of HIV- and CMV-responding $CD8^+$ T cells did not differ substantially across the groups, with the exception of a slightly larger Gag-specific response in CP compared to HAART-suppressed (**Fig. 16A**). This was in agreement with previous studies that compared magnitudes of HIV-specific cells in controlled and progressive infection (Betts et al., 2006; Hersperger et al., 2010; Migueles et al., 2002). The differentiation states of Gag-, Nef-, or CMV-specific cells between groups also were not significantly different (**Fig. 16 B and C**; Nef- and CMV-specific data not shown). This was in contrast to previous reports indicating HIV-specific cells from CP have a less differentiated phenotype compared to EC (Betts et al., 2006; Migueles et al., 2002; Precopio et al., 2007).

When we determined the relative contributions of CD107a, IFNγ, and MIP-1α to the total virus-specific $CD8^+$ T cell responses we found no differences between the groups (**Fig. 16 D and E**). Similar to previous reports, the responses were dominated by degranulating cells and/or cells that produced MIP-1α (Betts et al., 2006; Hersperger et al., 2010). This was true for Gag-, Nef-, and CMV-specific $CD8^+$ T cells (Nef- and

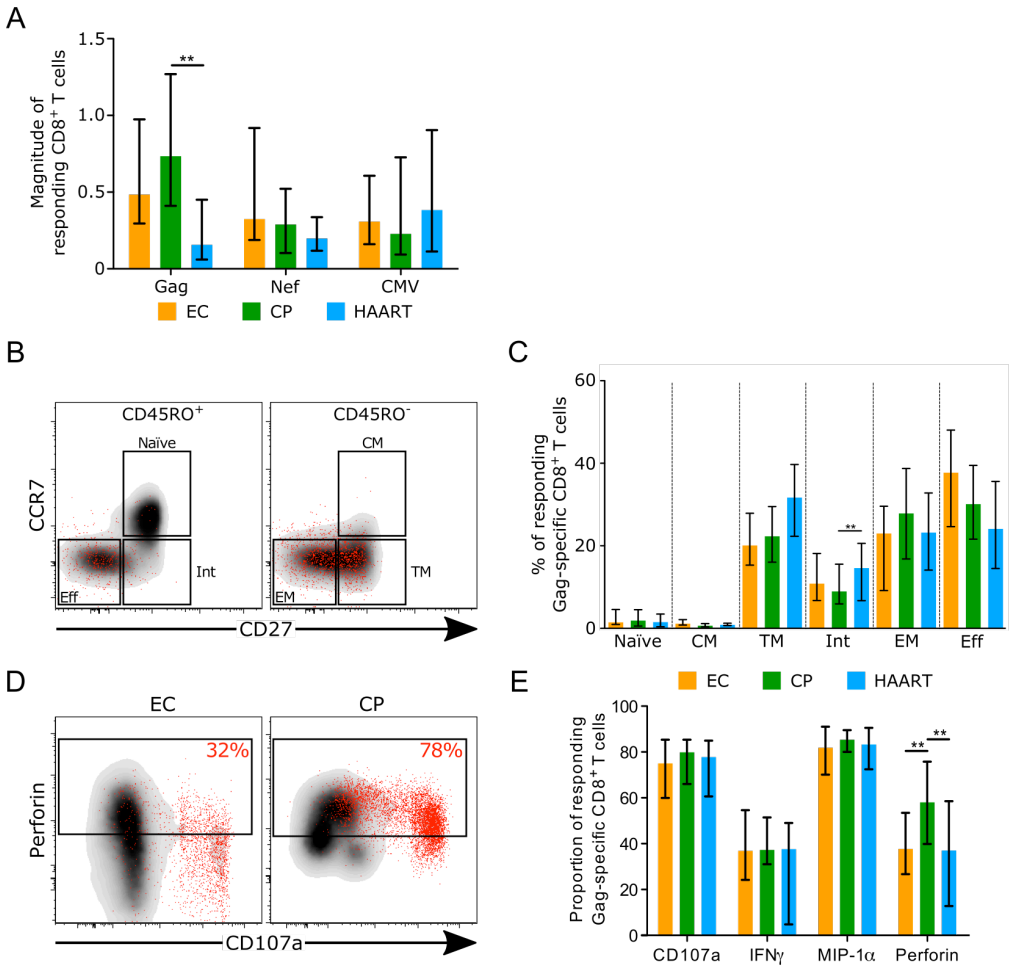

Figure 16. Magnitude and functionality of HIV-specific CD8$^+$ T cell responses. (**A**) (**A**) Frequency of Gag-, Nef-, and CMV-specific CD8$^+$ T cells within the memory CD8$^+$ T cell pools of EC (yellow), CP (green), and HAART (blue) as determined by measurement of degranulation (CD107a), IFN-γ expression, or MIP-1α expression in response to peptide stimulation. (**B**) Representative flow cytometric plots of CD45RO, CCR7, and CD27 profiles for Gag-specific CD8$^+$ T cells (red) overlaid on total CD8$^+$ T cells (black) for one EC. (**C**) Memory distributions for responding Gag-specific CD8$^+$ T cells as determined by CD45RO, CCR7, and CD27 staining for all EC, CP, and HAART. (**D**) Representative flow cytometric plots of perforin for Gag-specific CD8$^+$ T cells (red) overlaid on total memory CD8$^+$ T cells (black) for one EC and one CP. Percentages represent proportions of responding Gag-specific cells that express perforin for each donor. (**E**) Proportions of total responding Gag-specific CD8$^+$ T cells that degranulated (CD107a) or expressed IFNγ, MIP-1α, or perforin. Cells had to express at least one other function in addition to perforin to be considered Gag-specific. ** denotes a *P* value < 0.01. Statistics based on a Kruskal-Wallis test followed by Dunns post test for multiple comparisons.

CMV-specific data not shown). We did, however, find differences in the capacities of responding virus-specific cells to express perforin (**Fig. 16 D and E**). Similar to total memory $CD8^+$ T cells, there were greater frequencies of $perforin^+$ Gag-specific $CD8^+$ T cells for progressors compared to controllers. This difference was also observed for Nef-specific $CD8^+$ T cells, while CMV-specific cells from progressors trended toward being more $perforin^+$ but did not reach significance (data not shown). These data demonstrate that whereas the magnitudes and differentiation states of virus-specific $CD8^+$ T cells are similar between groups the functional quality of the responses is different, with greater potential cytolytic capacity in progressors.

Perforin is expressed by HIV-specific $CD8^+$ T cells from CP independent of T-bet and Eomes expression pattern

We recently demonstrated that responding HIV-specific $CD8^+$ T cells are predominantly $T\text{-}bet^{Hi}Eomes^+$ or $T\text{-}bet^{Lo}Eomes^+$ during acute HIV infection (Demers et al., 2016). Cells are evenly divided between these two expression patterns during the earliest phase of infection but the proportion of $T\text{-}bet^{Lo}Eomes^+$ cells increases significantly over time. As expected based on these previous findings, upon examination of T-bet and Eomes expression patterns for chronically infected individuals we found the majority of responding HIV-specific $CD8^+$ T cells were either $T\text{-}bet^{Hi}Eomes^+$ or $T\text{-}bet^{Lo}Eomes^+$, with cells skewing more towards the $T\text{-}bet^{Lo}Eomes^+$ phenotype (**Fig. 17 A and B**, Nef-specific data not shown). There was also a larger frequency of $T\text{-}bet^-Eomes^+$ cells than we previously observed during acute infection. CMV-specific cells were also mostly $T\text{-}bet^{Hi}Eomes^+$ or $T\text{-}bet^{Lo}Eomes^+$, but unlike HIV-specific cells, were skewed more towards

the T-bet^{Hi}Eomes^{+} phenotype and had few T-bet^{-}Eomes^{+} cells (data not shown). Notably, there were no major differences in any of the virus-specific CD8^{+} T cell T-bet and Eomes subset distributions between groups.

During acute HIV-infection, both T-bet^{Hi}Eomes^{+} and T-bet^{Lo}Eomes^{+} HIV-specific CD8^{+} T cells were capable of expressing perforin after short-term *in vitro* stimulation (Demers et al., 2016). As disease progressed, the capacity to express perforin was retained by T-bet^{Hi}Eomes^{+} cells and lost by T-bet^{Lo}Eomes^{+} cells. This along with other recent studies that examined T-bet and/or Eomes expression in HIV chronically infected individuals suggested high levels of T-bet expression were necessary for the maintenance of a high quality CD8^{+} T cell responses to HIV infection (Buggert et al., 2014; Hersperger et al., 2011). However, as noted above, we did not see any differences in the distributions of virus-specific CD8^{+} T cells across T-bet and Eomes subsets for CPs versus ECs, despite CD8^{+} T cells from CPs having more perforin expression. We therefore compared the functional capacities of HIV-specific CD8^{+} T cell T-bet and Eomes coexpression subsets across the groups. When we assessed CD107a, IFNγ, or MIP-1α we did not find any major differences between groups, *i.e.* cells with the same T-bet and Eomes coexpression profile from each group had similar proportions of cells expressing each of the three functions (**Fig. 17C**). Perforin expression, on the other hand, was significantly different between groups (**Fig. 17D**). Similar to total memory CD8^{+} T cells, for all groups the capacity of HIV-specific CD8^{+} T cells to express perforin decreased with lower levels of T-bet and absence of Eomes. However, progressors had greater frequencies of perforin^{+} CD8^{+} T cells across almost all T-bet and Eomes subsets, including cells without detectable levels of either transcription factor. Collectively, these

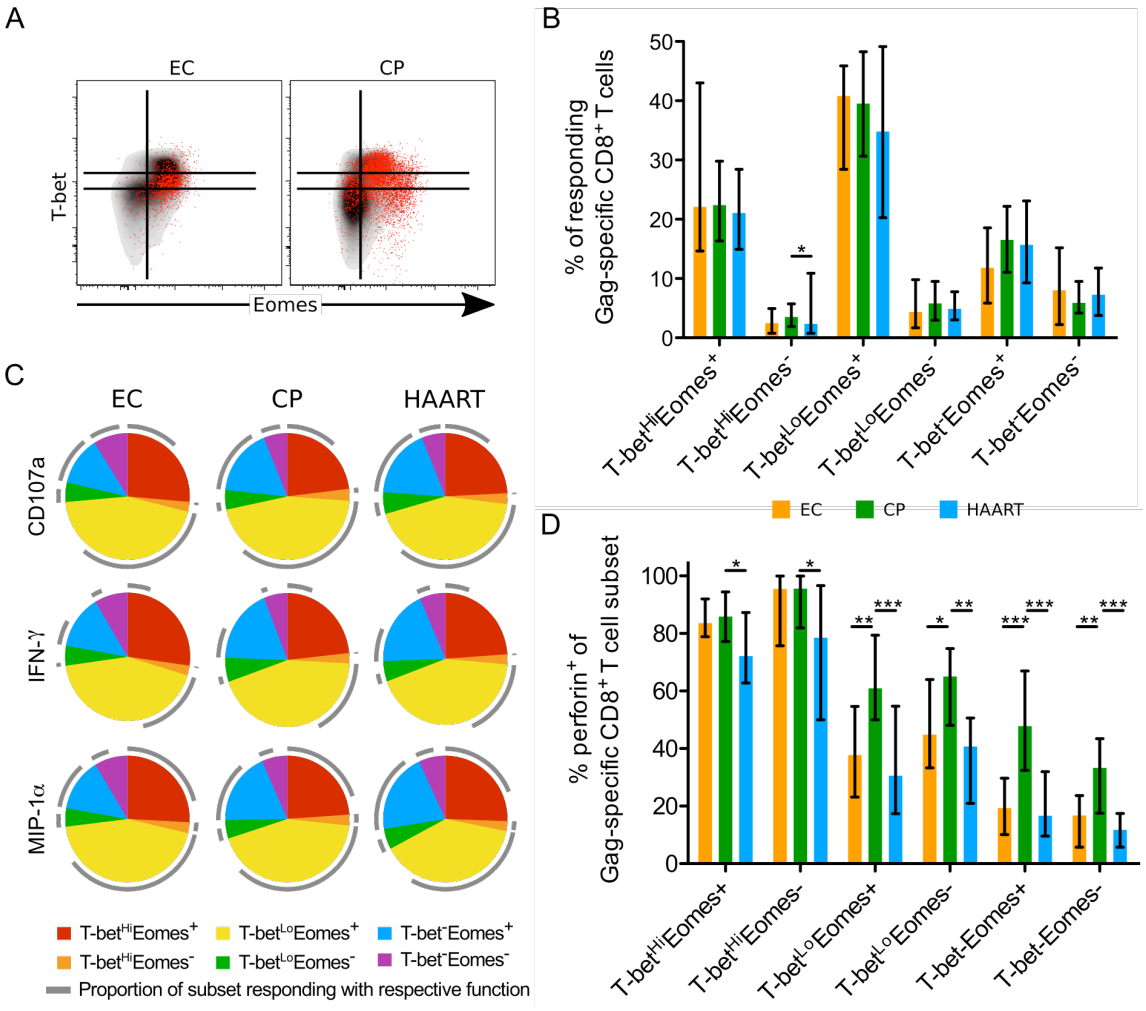

Figure 17. Proportion positive and functionality of HIV-specific CD8$^+$ T-bet and Eomes subsets. (**A**) Representative flow cytometric plot of T-bet and Eomes expression for Gag-specific CD8$^+$ T cells (red) overlaid on total memory CD8$^+$ T cells (black) from one EC and one CP. (**B**) T-bet and Eomes coexpression subsets for Gag-specific memory CD8$^+$ T cells for all EC (yellow), CP (green), and HAART (blue). (**C**) The average Gag-specific CD8$^+$ T cell T-bet and Eomes coexpression profile is shown for EC, CP, and HAART with the proportions of each subset that degranulated (CD107a) or expressed IFNγ, MIP-1α depicted by gray arcs. There were no statistically significant differences in the expression of these three functions by the respective T-bet and Eomes subsets from each group. (**D**) Proportions of Gag-specific CD8$^+$ T cell T-bet and Eomes subsets that express perforin. Cells had to express at least one other function in addition to perforin to be considered Gag-specific. * denotes a P value < 0.05, ** denotes a P value < 0.01, and *** denotes a P values < 0.001. Statistics based on a Kruskal-Wallis test followed by Dunns post test for multiple comparisons.

data provide evidence of a global dysregulation of perforin expression by $CD8^+$ T cells during uncontrolled chronic HIV infection.

Discussion

There is strong evidence to suggest effector $CD8^+$ T cell responses are important for the control of HIV infection. In this study, we examined the relationships between perforin, T-bet, and Eomes expression in $CD8^+$ T cells from individuals who differentially controlled HIV replication. We found both total memory and HIV-specific $CD8^+$ T cells from CP had greater capacities to express perforin compared to EC or HAART-suppressed individuals. Conversely, T-bet and Eomes, both transcriptional regulators of effector $CD8^+$ T cell differentiation and function (Cruz-Guilloty et al., 2009; Hersperger et al., 2011; Intlekofer et al., 2005; Makedonas et al., 2010; Pearce et al., 2003; Sullivan et al., 2003), were not differentially expressed between groups. Rather, perforin expression was directly associated with both T-bet and Eomes during controlled infection while its expression by $CD8^+$ T cells from CP appeared to be partially dysregulated such that cells with low or no expression of T-bet or Eomes were also able to express this effector molecule.

$CD8^+$ T cells kill virally infected targets primarily through the release of granules containing granzyme B and perforin (Barry and Bleackley, 2002; Podack, 1989). *In vivo* control of HIV viremia has previously been associated with the ability of $CD8^+$ T cells from chronically HIV-infected donors to upregulate these cytotoxic molecules after *in vitro* culture or following brief stimulation directly *ex vivo* (Hersperger et al., 2010; Migueles et al., 2008). Enhanced *in vitro* cytolytic capacity has also been linked to higher

levels of perforin expression by $CD8^+$ T cells (Migueles et al., 2008). In the current cohort, we observed greater frequencies of perforin$^+$ $CD8^+$ cells in CPs compared to ECs. This would suggest $CD8^+$ T cells from the group of CPs studied here should provide superior control over HIV replication. Although we did not conduct *in vitro* killing assays, the fact that we observed a direct correlation between the frequency of perforin$^+$ $CD8^+$ T cells and viral loads in CPs suggests a different relationship exists in these donors. It is possible we are measuring pre-formed perforin that, in the case of HIV-specific $CD8^+$ T cells from CPs, is not released upon activation. This would be consistent with one study that suggested $CD8^+$ T cells from CP have the capacity to express perforin but are unable to degranulate efficiently (Sakhdari et al., 2012). However, we found no difference in the ability of HIV-specific $CD8^+$ T cells from EC or CP to degranulate here. Alternatively, this discrepancy might arise from the use of Gag and Nef potential T cell epitopes rather than autologous virus for *ex vivo* stimulation. Potential T cell epitopes may not be representative of the protein sequences found in autologous HIV from each donor and therefore responses elicited in our *in vitro* assays could be qualitatively different from those taking place *in vivo*. Why this difference would be specific to CP donors is unclear. *In vitro* killing assays using autologous HIV-infected target cells would be necessary to determine the true cytolytic capacity of the $CD8^+$ T cell populations from each of the groups. Whatever the case, these data suggest that while perforin is a necessary component of an effective antiviral $CD8^+$ T cell-mediated immune response, $CD8^+$ T cell perforin content alone is not sufficient to predict *in vivo* control (Norstrom et al., 2012).

There has been increasing interest in the determinants of $CD8^+$ T cell differentiation and function (Glimcher et al., 2004; Kaech and Cui, 2012). T-bet and Eomes have been shown to be important regulators of the development of effector $CD8^+$ T cells in both mice and humans (Cruz-Guilloty et al., 2009; Hersperger et al., 2011; Joshi et al., 2007; Makedonas et al., 2010; McLane et al., 2013; Pearce et al., 2003; Pipkin et al., 2010; Sullivan et al., 2003; van Aalderen et al., 2015). Analysis of T-bet and Eomes in the context of several different viral infections in humans has demonstrated a variety T-bet and Eomes expression patterns associated with the $CD8^+$ T cell responses. EBV-specific $CD8^+$ T cells express T-bet and Eomes during the acute phase of infection but lose expression of both during convalescence (Greenough et al., 2015). Ticke-borne encephalitis virus-specific $CD8^+$ T cells also express both transcription factors during acute infection, but Eomes expression is gradually lost and T-bet maintained as the virus is cleared. During CMV or HCV infections $CD8^+$ T cells expressing T-bet are associated with control whereas expression of Eomes or expression of neither transcription factor is associated uncontrolled infection (Hertoghs et al., 2010; Kurktschiev et al., 2014; Paley et al., 2012; Popescu et al., 2014). Collectively, these studies suggest a link between controlled infection and the expression of T-bet, whereas Eomes is differentially associated with outcome. The disparate associations of Eomes with outcome may indicate Eomes plays a larger role in determining the fates of cells following the acute phase of resolved and unresolved infections than it does in driving the effector responses early after infection (Doering et al., 2012).

Expression of T-bet and Eomes has recently been described during acute and chronic HIV infection. Higher levels of T-bet expression by $CD8^+$ T cells were associated

with increased functionality, including a greater capacity to upregulate perforin, and control of viral replication in EC (Buggert et al., 2014; Demers et al., 2016; Hersperger et al., 2011). Low levels of T-bet and high levels of Eomes expression were associated with lower overall functionality and exhaustion of $CD8^+$ T cells in CP. Given the increased frequency of $perforin^+$ $CD8^+$ T cells we observed for CP in the current cohort we anticipated that these individuals would also have increased levels of T-bet. This was not the case, however, as T-bet levels were not different between the groups for either total memory or HIV-specific $CD8^+$ T cells. Nor did Eomes appear to be acting in a compensatory fashion to drive perforin expression as might have been predicted from murine models (Cruz-Guilloty et al., 2009; Pearce et al., 2003). Instead we found increased frequencies of $perforin^+$ cells across all T-bet and Eomes coexpression patterns in CP relative to controllers, suggesting perforin expression is dysregulated during chronic progressive infection.

What is driving perforin expression if not T-bet or Eomes? Perforin expression was directly associated with plasma antigen levels here, and others have shown HIV viral load directly correlates with generalized activation of the T cell compartment (Mellors et al., 2007). The aberrant expression of perforin by cells expressing low or no T-bet or Eomes therefore might reflective of a state of heightened activation and inflammation. To this end, IL-15 has been shown to increase the activation of $CD8^+$ T cells during chronic HIV infection and is known to increase perforin expression *in vitro* (Bastidas et al., 2014; White et al., 2007; Younes et al., 2016). IL-15 likely induces perforin through a STAT-5 mediated mechanism (Grange et al., 2013; Johnston et al., 1995; Lin et al., 2012; Pipkin et al., 2010; Verdeil et al., 2006), but whether it can do so in the absence of T-bet and

Eomes remains unclear. IFNα, another cytokine associated with inflammation, has also been show to increase perforin expression, but at the translational level independent of new transcription (Kohlmeier et al., 2010). Lastly, metabolic cues from the microenvironment may lead to heightened perforin expression by $CD8^+$ T cells in the absence of T-bet and Eomes as perforin is positively regulated and T-bet and Eomes are negatively regulated under hypoxic conditions such as those found in inflamed tissues (Doedens et al., 2013). Whichever of these is taking place, a non-specific mechanism of perforin upregulation would fit with its dissociation from T-bet as T cell receptor stimulation should induce T-bet expression (Szabo et al., 2000). It may also explain why cells that express perforin directly *ex vivo* are not able to control viral replication *in vivo*: lack of the transcription factors necessary to drive efficient perforin expression in an antigen-specific response are ill-equipped to mount a durable effector response to infection.

Whether perforin expression is increased as a result of specific or non-specific activation, one notable observation to emerge from these results is that maintenance of bulk perforin$^+$ $CD8^+$ T cell subsets with T-bet and Eomes expression profiles similar to EC (i.e. high levels of T-bet and Eomes expression) correlated with better *in vivo* control of HIV replication. It is important to acknowledge that the associations between these subsets and lower viral load may be a cause or an effect. However, a recent $CD8^+$ T cell depletion study in the nonhuman primate model of SIV infection demonstrated a positive correlation between the frequency of T-bet$^+$ $CD8^+$ T cells prior to depletion and the fold-increase in plasma viral load post-depletion (Chowdhury et al., 2015). While this study did not differentiate T-bet levels or its association with perforin expression, it does

support the idea of a causal relationship between T-bet and viral control. Further work is needed to determine the extent to which perforin is regulated by T-bet and Eomes in human $CD8^+$ T cells and to find other regulatory factors that might be involved in its expression. Collectively, these results suggest that perforin expression, while important for antiviral $CD8^+$ T cell responses in general, on its own may not be sufficient to define a robust effector $CD8^+$ T cell response in the context of chronic HIV infection. Perforin expression in combination with high levels of T-bet and Eomes provides a better definition of effective $CD8^+$ T cells. Vaccine or cure strategies that can induce all three may be necessary in order to drive HIV-specific $CD8^+$ T cell responses capable of clearing virus-infected cells.

CHAPTER 4

EPILOGUE

Implications and considerations

Despite three decades of research, the HIV/AIDS pandemic remains a significant global health challenge. Optimized antiretroviral therapy (ART) has made it possible to achieve durable control of HIV replication, thereby preventing onset of AIDS and reducing overall mortality. However, there are significant challenges to the sustainability of lifelong treatment including the economic burden, drug availability, and potential drug toxicities. In addition, ART does not eradicate the latent HIV reservoir so that treatment interruption in most cases leads to viral rebound. This combination of factors points to the continued need to identify an effective means of controlling HIV in the absence of therapy. Several lines of evidence indicate $CD8^+$ T cells are critical for both the initial and long-term control of HIV replication, with HIV-specific $CD8^+$ T cell cytotoxic capacity, and the cytolytic molecule perforin in particular, being associated with enhanced viral suppression (Borrow et al., 1994; Hersperger et al., 2010; Koup et al., 1994; Migueles et al., 2008; Saez-Cirion et al., 2007). Recent efforts to elucidate the regulatory elements involved in driving cytotoxic $CD8^+$ T cell differentiation and function identified the T-box transcription factors T-bet and eomesodermin (Eomes) as potential targets for manipulation that might induce more robust cytotoxic $CD8^+$ T cell responses to HIV infection. With the work described in Chapters 2 and 3 here, we sought to expand on these earlier findings by examining $CD8^+$ T cell perforin expression and its interplay with T-bet and Eomes at various stages of HIV infection in the hope of gaining

insights into the molecular regulation of cytotoxic $CD8^+$ T cell responses that might inform strategies for sterilizing and/or functional cures.

Although there is clear evidence from humans and nonhuman primates that $CD8^+$ T cells contribute to the initial resolution of peak viremia during acute HIV and SIV infections, respectively, relatively little was known about the functional status of the responding cells until recently. Reports from Ferrari *et al.* and Ndhlovu *et al.* demonstrated that HIV-specific $CD8^+$ T cells have limited functionality during the earliest phase of infection with the majority of cells degranulating (expressing CD107a) or upregulating the β-chemokine MIP-1β (Ferrari et al., 2011; Ndhlovu et al., 2015). While the high degree of degranulating cells observed by both groups was suggestive of cytolytic responses, there are instances in which CD107a may overestimate or underestimate the true cytolytic potential of $CD8^+$ T cells (Makedonas et al., 2009; Wolint et al., 2004). As such, we sought to determine if HIV-specific $CD8^+$ T cells were capable of upregulating perforin during acute HIV-infection and how its initial expression and maintenance was related to T-bet and/or Eomes expression. To this end, we assemble of a cohort of untreated individuals experiencing acute/early primary HIV infection and examined the global and HIV-specific responses longitudinally.

We made three profound observations regarding $CD8^+$ T cell responses to acute HIV infection. First, both total and HIV-specific $CD8^+$ T cells were indeed capable of upregulating perforin at the outset of infection, but HIV-specific $CD8^+$ T cells rapidly lost this capacity following the resolution of peak viremia. Second, during the earliest responses, perforin expression by $CD8^+$ T cells was not restricted to any one T-bet and Eomes coexpression subset and even total memory $CD8^+$ T cells with no discernible T-

bet or Eomes expressed perforin. However, maintenance of perforin expression by HIV-specific $CD8^+$ T cells was largely restricted to cells that were T-$bet^{Hi}Eomes^+$. Lastly, HIV-specific $CD8^+$ T cells were equally distributed between T-$bet^{Hi}Eomes^+$- and T-$bet^{Lo}Eomes^+$-expressing subsets initially but became progressively more T-$bet^{Lo}Eomes^+$ over time. Together these data suggest a robust effector $CD8^+$ T cell response takes place following HIV infection with maintenance of this response dependent upon that ratio of T-bet and Eomes expression on a per-cell basis. High levels of T-bet relative to Eomes are necessary for the expression of perforin whereas cells with lower levels of T-bet remain functional but are have reduced cytotoxic capacity. Unfortunately, the relatively narrow range of set point viral loads within the cohort as well as the lack of any donors who spontaneously controlled HIV replication to undetectable levels limited our ability to determine if any specific functional or T-bet and Eomes expressing subset was associated with greater *in vivo* control.

Future studies comparing $CD8^+$ T cell responses from HIV acutely infected progressors and elite controllers, either in humans or using a nonhuman primate model, may provide insight into differences that render controller $CD8^+$ T cell responses protective. What these differences might be during the earliest phase of infection is uncertain since the initial HIV-specific $CD8^+$ T cell responses of progressors have a phenotype (T-bet^{Hi}) and function ($perforin^+$) we would predict to be protective. The difference may be a matter of quantity of HIV-specific cells, in which case controllers would be predicted to have a greater overall magnitude of cells expressing high levels of T-bet and perforin. Alternatively, it may be that controller $CD8^+$ T cells are restricted to more conserved epitopes which prevents viral escape and allows controllers to maintain a

strong and effective response. Control of viral replication could, in turn, prevent over-stimulation of the $CD8^+$ T cells and thereby decrease the chance of driving the exhaustion of the response. These are not necessarily mutually exclusive and it could be a combination of theses factors that permit EC $CD8^+$ T cells to continue to express T-bet and perforin and ultimately control HIV replication into chronic infection.

We next sought to determine the association between perforin expression and T-bet and Eomes for $CD8^+$ T cells from individuals with differential abilities to control HIV replication *in vivo* during chronic infection. Previous work has shown that HIV-specific $CD8^+$ T cells from individuals who spontaneously control HIV to undetectable levels in the absence of ART (elite controllers or EC) generally have a greater capacity to upregulate perforin *in vitro* than cells from individuals with chronic progress HIV infection [chronic progressors or CP](Hersperger et al., 2010; Migueles et al., 2008). Work from our lab demonstrated that HIV-specific $CD8^+$ T cells from EC also tended to express higher levels of T-bet compared to cells from CP and T-bet expression level directly correlated with the ability of cells to upregulate perforin (Hersperger et al., 2011). Another more recent report by Buggert *et al.* demonstrated an inverse relationship between perforin and Eomes expression in $CD8^+$ T cells from CP (Buggert et al., 2014). This study did not include EC, however, and so could not determine if Eomes expression further differentiated $CD8^+$ T cell responses from individuals with disparate clinical outcomes. However, based on these previous studies, we hypothesized that expression of high levels of T-bet relative to Eomes expression would be associated with a more cytotoxic $CD8^+$ T cell response marked by a greater capacity to express perforin whereas

low T-bet levels relative to Eomes would result in inferior $CD8^+$ T cell responses with little or no cytotoxic function (Figure 18).

In contrast to previous chronic cohorts, for the cohort examined in Chapter 3 we found that CP had greater frequencies of both total memory and HIV-specific $perforin^+$ $CD8^+$ T cells than EC or ART-suppressed individuals (HAART). In addition, whereas perforin expression in EC and ART donors strongly correlated with T-bet and Eomes expression, $CD8^+$ T cells from CP expressed perforin irrespective of their T-bet or Eomes content. We did, however, observe that CP with greater frequencies of the $perforin^+$ $CD8^+$ T cell T-bet and/or Eomes expressing subsets found in EC (*i.e.* $T\text{-}bet^{Hi}$, $Eomes^+$, or $T\text{-}bet^{Hi}Eomes^+$) tended to have lower viral loads while the frequency of $T\text{-}bet^-Eomes^-$ $perforin^+$ $CD8^+$ T cells correlated directly with viremia. These data, in combination with data from the acute cohort and previous reports, imply better control of *in vivo* HIV replication can be achieved when $perforin^+$ $CD8^+$ T cells with high levels of T-bet are maintained.

It is important to acknowledge that these observations are correlational and as such the relationship between T-bet and viral loads as well as the maintenance of the relationship between T-bet and perforin may be either the cause or the effect of control of HIV replication. However, data from other human infections including CMV and HCV found similar positive associations between T-bet expression and favorable clinical outcomes (Kurktschiev et al., 2014; Paley et al., 2013; Popescu et al., 2014). In addition, a recent $CD8^+$ T cell depletion study in SIV-infected rhesus macaques demonstrated that macaques with higher frequencies of $T\text{-}bet^+$ $CD8^+$ T cells pre-depletion experienced greater increases in plasma viremia post-depletion (Chowdhury et al., 2015). Although it

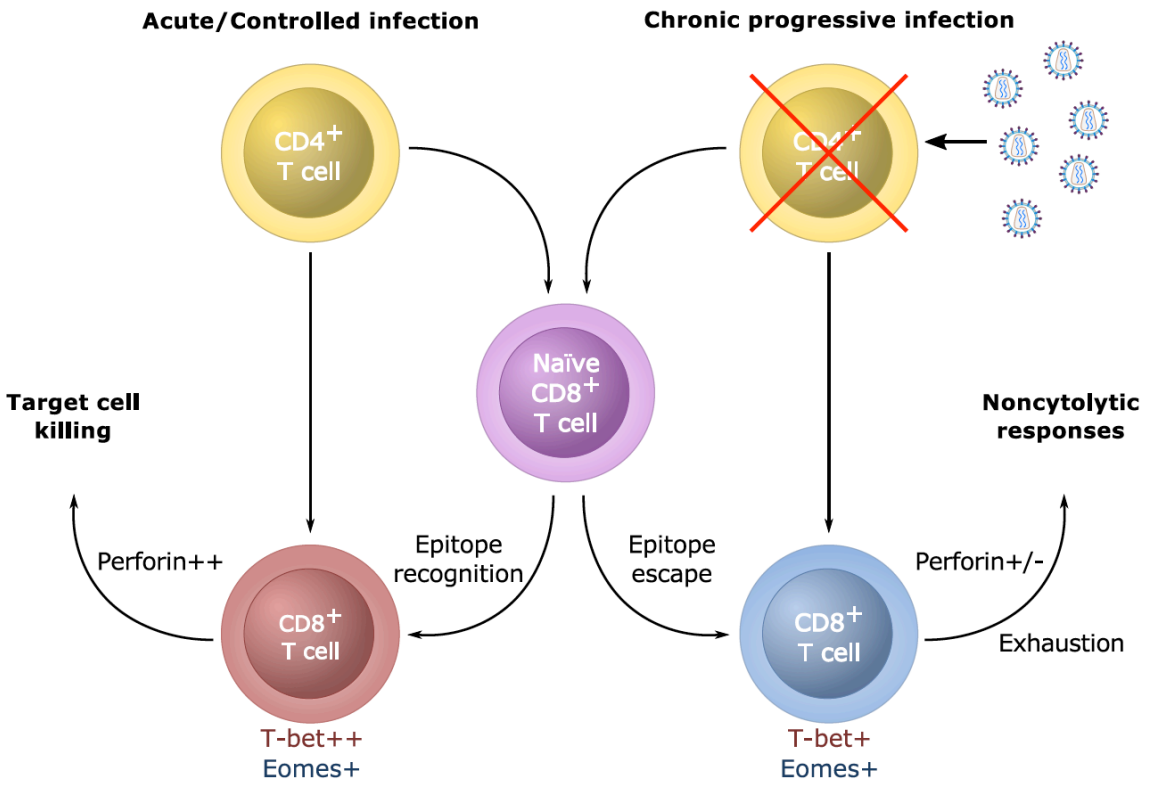

Figure 18. Model of CD8$^+$ T cell responses in the context of acute/controlled and chronic HIV infection. During acute/controlled HIV infection, CD4$^+$ T cells are present to drive CD8$^+$ T cell differentiation both directly and indirectly through the production of cytokines and licensing of dendritic cells. Viral escape within CD8$^+$ T cell-restricted epitopes has not occurred and infected cells provide a strong stimulation via the peptide/MHC-T cell receptor interaction. There is also little or no exhaustion within the CD8$^+$ T cell compartment to dampen the ability of cells to receive and propagate stimulatory signals. Together, these factors allow strong stimulation of HIV-specific CD8$^+$ T cells resulting in high levels of T-bet expression and thereby the ability to upregulate perforin to kill infected target cells. During chronic progressive infection, HIV depletes the CD4$^+$ T cell pool diminishing the support necessary for driving effector CD8$^+$ T cell responses; viral escape occurs resulting in poor or complete loss of recognition of infected target cells; increased exhaustion dampens stimulatory signals. The overall effect of these factors, either alone or in concert, is that responding CD8$^+$ T cells are unable to express high levels of T-bet resulting in the inability of these cells to express perforin. Responding cells instead realize noncytolytic mechanisms with inferior capacity to control viral replication.

does not rule out the possibility that T-bet and perforin are themselves correlates of some other $CD8^+$ T cell functional or regulatory capacity not assessed here, collectively these data strongly indicate T-bet plays an important role in driving effective $CD8^+$ T cells responses.

This work also raises several important questions. For one, the initial wave of HIV-specific $CD8^+$ T cells appear to be highly cytotoxic but subsequent responding cells, be they progeny of the first wave or *de novo*, lose the ability to express perforin in association with a concomitant loss of the ability to express high levels of T-bet. Why do cells lose the ability to express these effector and regulatory molecules and how can they be sustained? Additionally, even if they could be sustained would the cells be any more effective at suppressing viral replication? There may be several factors at play that ultimately result in the decline of cytotoxic $CD8^+$ T cells responses. IL-2 helps drive effector $CD8^+$ T cell differentiation and loss of IL-2-producing $CD4^+$ T cells during acute infection may result in impaired development of cytotoxic $CD8^+$ T cells response. Expression of the inhibitory receptor PD-1 was recently shown to be increased on total $CD8^+$ T cells during the acute phase of HIV infection and expression likely increases on HIV-specific cells as infection progresses. PD-1 attenuates signaling through the T cell receptor (TCR) in a dose-dependent manner with intermediate levels sufficient to prevent cytotoxicity and high levels required to inhibit β-chemokine production (Wei et al., 2013). Increased expression of PD-1 on responding $CD8^+$ T cells would therefore be consistent with the functional changes observed with time from infection for our cohort. It would also be consistent with the reduced levels of T-bet expression given that T-bet is induced by TCR stimulation (Szabo et al., 2000). This suggests PD-1 blockade during

acute infection may allow for continued cytotoxic responses. This, however, is not taking into consideration the effects of viral escape mutations. The dynamics of loss of perforin expression by HIV-specific $CD8^+$ T cells during acute HIV infection shown in Chapter 2 coincides with the emergence of $CD8^+$ T cell escape variants (Goonetilleke et al., 2009b; Liu et al., 2013). As such the decline in cytotoxic responses might reflect reduced or complete loss of recognition of epitopes by $CD8^+$ T cells with high functional avidity. In this instance neither the maintenance of $CD4^+$ T cell help nor PD-1 blockade would likely be sufficient to drive continuous cytotoxic responses without also generating new $CD8^+$ T cell responses to alternate and more conserved targets. This problem was recently highlighted in a report from Deng *et al.* showing that HIV-specific $CD8^+$ T cells from ART-suppressed chronically infected individuals are largely incapable of recognizing autologous virus following treatment of primary cells with a latency reversing agent (Deng et al., 2015).

Another issue is the dissociation of perforin expression from T-bet and Eomes expression we observed for total memory $CD8^+$ T cells in acute infection and both total memory and HIV-specific $CD8^+$ T cells during chronic progressive infection. For total $CD8^+$ T cells, the simplest explanation for the increased perforin expression in the absence of T-bet and Eomes is that it is reflective of a population of HIV-specific cells that once did express T-bet and/or Eomes, but subsequent lose of antigen stimulation resulted in downregulation of T-bet and Eomes while granules already loaded with perforin were retained. However, this explanation does not fit for HIV-specific $CD8^+$ T cells given the similar propensities of $CD8^+$ T cell from both EC and CP to degranulate and thereby presumably release any preformed perforin. Another possibility is that

perforin is being induced in a non-specific manner whereby regulatory elements other than T-bet or Eomes drive its expression or its expression is controlled at the translational level rather than the transcriptional level. Several different signal transducer and activator of transcription (STAT) molecules have been linked to perforin expression and some of them are activated by cytokines (*e.g.* INF-α and IL-15) that are elevated during HIV infection (Demers et al., 2013; Stacey et al., 2009). IFN-α may also stabilize perforin mRNA thereby increasing perforin protein expression by enhancing translation (Kohlmeier et al., 2010). Non-specific activation would explain the dissociation observed for both total memory and HIV-specific $CD8^+$ T cells. It would also be consistent with the larger increases of T-bet^-Eomes^- cells we observed for total HLA-DR^+ $CD8^+$ T cells compared to $perforin^+$ $CD8^+$ T cells following vaccination with vaccinia virus or live attenuated yellow fever virus in Chapter 2. This suggests increased perforin expression in the absence of T-bet or Eomes is a result of generalized immune activation. To determine if T-bet and/or Eomes expression is required for perforin expression by human $CD8^+$ T cells it will be necessary to perform *in vitro* experiments in which T-bet, Eomes, or both are knocked down or out in primary cells prior to stimulation via the T cell receptor or non-specific stimulation via exposure to cytokines. Such studies could help determine at which stage of cytotoxic $CD8^+$ T cell development these transcription factors are most important and if there are alternative routes to driving cytotoxic responses by non-antigen-specific means.

The work presented here in combination with previous reports suggest a robust effector $CD8^+$ T cell response that is durable, rapid, and targets highly conserved viral epitopes is required to achieve control over HIV replication. The question then becomes

how such a response can be induced *in vivo*. Several cytokines have shown promise in their capacities to enhance effector $CD8^+$ T cell responses and may prove important for vaccine or therapeutic strategies (Reuter et al., 2012). However, these would likely need to be used alongside other modalities necessary for the expansion of cells with appropriate specificity. To this end, a number of HIV vaccines have been tested, including DNA vectors, recombinant proteins or viruses, DNA vectors, and dendritic cells presenting autologous antigens (Autran et al., 2008; Buchbinder et al., 2008; Fauci et al., 2014; Fauci and Marston, 2015; Garcia et al., 2012; Robb and Kim, 2014). While vaccines generated or improved HIV-specific responses, almost all of these platforms are not persistent, producing antigens for a limited amount of time. As a result, when antigen disappears the vaccine-elicited T cells gain a central memory phenotype. While cells with this phenotype have high proliferative potential they lack appreciable levels of T-bet or perforin and are therefore incapable of rapid effector responses (Makedonas et al., 2010; McLane et al., 2013; van Aalderen et al., 2015). To date, the most efficacious vaccine model has been an attenuated Rhesus CMV-based vector that has been shown to be capable of completely clearing virus from SIV-infected macaques (Hansen et al., 2011; Hansen et al., 2013a). $CD8^+$ T cells were critical for control in this model and while the exact qualitative nature of the vaccine-elicited response has yet to be fully elucidated, the effector memory phenotype and capacity responding cells to degranulate is highly suggestive of a strong cytotoxic response (Hansen et al., 2011; Hansen et al., 2009). This combined with the broad specificity and non-classical restriction of the $CD8^+$ T cell response as well as the persistent nature of the CMV vector fulfills almost all of the requirements for a highly effective prophylactic or therapeutic vaccine strategy (Hansen

et al., 2013b; Hansen et al., 2016). It remains to be seen if regulatory issues surrounding the use of a CMV-based platform in humans can be overcome or if it will have similar efficacy outside of the rhesus model.

A final hurdle to achieving a fully protective response is the issue of compartmentalization of HIV and the antiviral $CD8^+$ T cells required to fight infection. T follicular helper cells serve as the primary reservoir for HIV and these cells are found in large numbers in specialized follicles within lymph nodes (Banga et al., 2016; Folkvord et al., 2005; Perreau et al., 2013). Several studies have now shown that these follicles constitute immune privileged sites from which $CD8^+$ T cells with cytolytic potential are largely excluded (Andersson et al., 1999; Connick et al., 2007; Folkvord et al., 2005; Shacklett et al., 2004). This issue was further highlighted by a recent report from Fukazawa *et al.* that demonstrated the viral reservoir is limited exclusively to follicles in rhesus macaques with elite controller status (Fukazawa et al., 2015). With the viral reservoir seeded in as little as three days after infection (Whitney et al., 2014), compartmentalization represents a significant barrier to any prophylactic or curative strategy. Thus, while we and others have begun to define some of the qualitative and regulatory properties of $CD8^+$ T cells from peripheral blood that correlate with *in vivo* protection it will be important to next determine if these same properties are reflective of protective responses within gut and lymphoid tissues.

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